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16805

Wallingford Poliomyelitis Virus: Another Strain of the Lansing Type, Infective in Rodents.*

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Since the original isolation by Armstrong[1] of poliomyelitis virus capable of infecting rodents, 4 other strains immunologically related to the Lansing virus and also infective in rodents have been established. One of these is the M.E.F.1. strain, isolated from the central nervous system (C.N.S.) of a fatal case in the British Middle East Forces in 1942.[2,3] Two others isolated by investigators at Yale are the S.K. and Phillips strains. The first was obtained from feces of a non-paralytic patient in New Haven in 1937[4,5] and the second was obtained from the C.N.S. of a fatal case in Egypt in 1943.[5] A fourth strain (W.W.)[6] was isolated from the blood-stream of a patient in New York in 1946, although such blood-stream isolations of poliomyelitis virus are very rare events.

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* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

[1] Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

[2] Van Rooyen, C. E., and Morgan, A. D., *Edinburgh Med. J.*, 1943, **50**, 705.

[3] Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *Science*, 1943, **98**, 452.

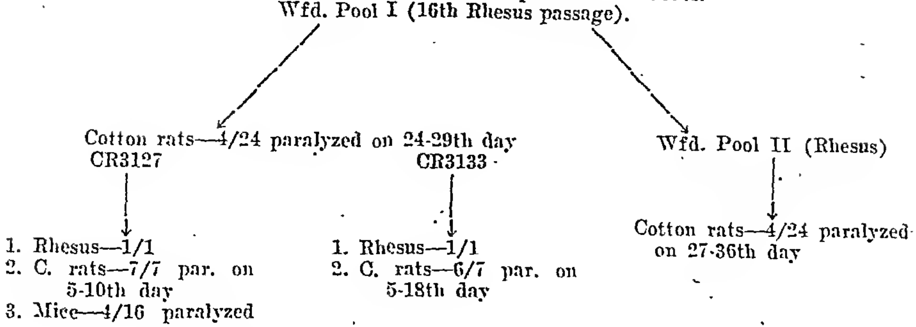
[4] Trask, J. D., Vignec, A. J., and Paul, J. R., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 147.

[5] Melnick, J. L., and Ward, R., *Fed. Proc.*, 1948, **7**, 308.

[6] Koprowski, H., Norton, T. W., and McDermott, W., *Pub. Health Rep.*, 1947, **62**, 1467.

CHART 1.

Passage of 10% Wfd. Virus Suspension in Rodents.


Wfd. Pool I (16th Rhesus passage).
Cotton rats—4/24 paralyzed on 24-29th day
CR3127
CR3133
1. Rhesus—1/1
2. C. rats—7/7 par. on 5-10th day
3. Mice—4/16 paralyzed
1. Rhesus—1/1
2. C. rats—6/7 par. on 5-18th day
Wfd. Pool II (Rhesus)
Cotton rats—4/24 paralyzed on 27-36th day

In the course of investigations of strain differences, in which it was clearly shown that three distinct types of poliomyelitis viruses can be identified, the suspicion arose that another strain used in this laboratory—the Wallingford (Wfd.)—might be immunologically related to Lansing virus. This strain was found to be unrelated to the Brunhilde virus, as will be shown, and since the Brunhilde strain is immunologically distinct from the Lansing, it was decided to compare the Wfd. with the Lansing strain. The Wfd. strain was isolated in California in 1934 by Trask and Paul and was soon described as a strain of unusual infectivity by intracutaneous inoculation in monkeys.[7] It was obtained in this laboratory in 1938 through the courtesy of the late Dr. James Trask, and was used in experiments to utilize its apparently high infectivity by the intracutaneous route until 1940. It was then in its sixteenth rhesus passage, having been maintained as an aqueous 20% suspension, stored in a dry-ice chest, since it was obtained in the form of glycerolated cord, in the eleventh rhesus passage, from Dr. Trask.

A pool of cords from 2 rhesus monkeys, both representing the sixteenth rhesus passage (Wfd. Pool I), and a second pool (Wfd. II) derived from 3 monkey cords infected with virus from Wfd. Pool I, were used for the rodent passage experiments. The Pool I suspension had been stored in a dry-ice chest from 1940 to 1948. Chart I summarizes the results of these rodent passages and the reverse passage back to rhesus monkeys.

It will be noted that in the first passage to cotton rats of Pool I and Pool II, not a single incubation period was less than 24 days, among 8 cotton rats paralysed. In contrast, cotton rats inoculated with material from 2 cotton rats, paralysed in the first rodent passage, had much shorter incubation periods (11 of 14 between 5 and 10 days). The cords of these 2 cotton rats each infected the single rhesus monkey inoculated therewith, and the one cotton rat cord inoculated intracerebrally in mice paralysed 4 of 16. The infected rhesus monkeys showed typical paralytic poliomyelitis and, as was the case with the cotton rats, exhibited typical poliomyelitis lesions in the brain and spinal cord.

Immunological relationship to Lansing virus. The Wfd. virus was shown to be immunologically closely related to the Lansing virus and unrelated to the Brunhilde virus (Table I). Four rhesus monkeys immunized intramuscularly with the active Brunhilde virus, and shown to be immune to intracerebral challenge with 10,000 PD 50 of the Brunhilde virus, according to the method previously described,[8] promptly succumbed to intracerebral challenge with 0.8 cc of 10^{-1} cord suspension of Wfd. Pool I. All 4 had severe paralytic poliomyelitis, confirmed by histopathological examination of the central nervous system.

Four rhesus monkeys similarly immunized with, and immune to, Lansing virus were also challenged in the same fashion with virus of Wfd. Pool I. Ten normal controls were simi-

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[7] Trask, J. D., and Paul, J. R., *J. Bact.*, 1936, **31**, 527.

[8] Morgan, I. M., *Am. J. Hyg.*, in press.

TABLE I.
Immunological Relationship of Wfd. Poliomyelitis Virus to Lansing and Brunhilde Strains.

	Inoculated intracerebrally with Wfd. virus	
Immunizing virus	Convalescent rhesus monkeys	Vaccinated monkeys shown to resist immunizing virus
Brunhilde	4/4	4/4
Lansing	0/3	1/4
Normal controls	10/10	

larly challenged. All 10 controls succumbed to poliomyelitis infection, whereas only one of the 4 Lansing-immune monkeys was paralysed. Since these immunized monkeys had, because of the summer vacation, been challenged 2½ months after their last experience with Lansing virus, it was thought that a drop in antibody titer might have accounted for the failure of the one monkey to resist. Accordingly, his serum was titrated against $10^{-2.5}$ (=10 LD_{50}) Lansing virus in mice, and was found to have a neutralizing titer of 1 in 300. Since it is known that at this level only about 50% of monkeys are protected, and that the level of serum antibody associated with practically complete intracerebral immunity is of the order of 1 in 1,000,[9] it is likely that complete immunity of the 4 Lansing-immune monkeys to Wfd. virus would have resulted if the animals had been challenged no longer than one month after the course of immunization. A quite comparable result was obtained by challenge of monkeys with Wfd. virus several months after recovery from attacks of poliomyelitis with Lansing and Brunhilde viruses, respectively (Table I).

Discussion. It is interesting that the Wfd. virus, along with another virus (McC.) isolated in the same epidemic from nasal washings of a non-paralytic patient,[10] and the Aycock-1920 strain, were all considered to be related to the S. K. virus, as determined by neutralization tests.[11] Since the S.K. virus is immunologically related to the Lansing virus, and infective in rodents, further evidence regarding the affinities of the McC. and Aycock-1920 strains would be of interest. Aside from these 2 viruses, it appears that at least 6 other established strains are either definitely or very likely closely related to the Lansing virus by immunological test. Of these, 5 have thus far been passaged in rodents.

On the basis of intracerebral neutralization tests in mice, in which it was found that convalescent serum of monkeys infected with M.E.F.2. virus (Egypt, 1942) neutralized Lansing virus and M.E.F.2. virus, Schlesinger, Morgan and Olitsky concluded that M.E.F.2. virus was serologically of the Lansing type.[3] Our own cross-immunity experiments, however, indicate that rhesus monkeys immunized with, and immune to, Lansing virus may still be infected with M.E.F.2. virus. On the contrary, animals immunized with, and immune to, Brunhilde virus (known to be unrelated to Lansing serologically and immunologically) are completely resistant to M.E.F.2. virus.[12] This virus, therefore, cannot be considered to be primarily of the Lansing type. As shown by Schlesinger, *et al.*, it is not infective in cotton rats. Since a third strain (M.E.F.6.) studied from the Egyptian outbreak in 1942 in the Middle East Forces by the same authors, was shown by them to be serologically unrelated to the Lansing virus, it appears not unlikely from their results and from ours that 3 distinct strains produced fatal illness in that outbreak. The first, M.E.F.1., is of the Lansing type, and is infective in rodents. The second, M.E.F.2., although serologically related to the Lansing virus, at least to some extent, is more closely related to a known non-Lansing strain, the Brunhilde, by cross-immunity experiments. The third, M.E.F.6., is apparently unrelated serologically to the Lansing strain, but more details of its affinities are needed before it can be stated that it is entirely unrelated to the Lansing.

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[9] Morgan, I. M., to be published.

[10] Paul, J. R., Trask, J. D., and Webster, L. T., *J. Exp. Med.*, 1935, **62**, 245.

[11] Trask, J. D., Paul, J. R., and Vignec, A. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 241.

[12] Bodian, D., Morgan, I. M., and Howe, H. A., *Am. J. Hyg.*, in press.

The 9. strains either demonstrated or thought to be of the Lansing type, are listed below:

Lansing	—1938—Mich.[1]	Passaged in rodents
M.E.F.1	—1942—Egypt[3]	
Y-S.K.	—1937—New Haven[5,11]	
Phillips	—1943—Egypt[5]	
Wfd.	—1934—Los Angeles[7]	
W.W.	—1946—N. Y.[6]	
M.V.	—1909-1914—N. Y.	Not passaged in rodents
McC.	—1934—Los Angeles[10] (?)	
Aycock	—1920—Vt.[11] (?)	

Three of the above strains (M.E.F.1., Wfd., M.V.) have been shown in this laboratory to be not only closely related to the Lansing, but unrelated to the Brunhilde virus by tests of vaccination-immunity. Seven other strains isolated in this country from 1939 to 1946, have been shown to be related to the Brunhilde virus by the same method.[12] It seems evident, therefore, that any virus shown either to be unrelated to strains of the Brunhilde type, or related to those of the Lansing type should be investigated with respect to possible rodent pathogenicity. Such tests should be done in cotton rats rather than in mice, because of the known greater susceptibility of the former to the Lansing virus,[13] and moreover a large number of cotton rats should be inoculated. Observations of inoculated cotton rats should be continued for at least 2 months, since, as shown by Armstrong,[1] by Schlesinger, *et al.*,[3] and by our results, incubation periods may be of long duration.

It is interesting that of the six strains known to be closely related immunologically to the Lansing strain, five have been passaged in rodents. This fact leads to the strong suspicion that rodent pathogenicity is closely linked to the specific antigenic structure of this group of viruses. At first sight the MV strain appears to be an exception, but since it has had a long series of monkey passages since its isolation some 40 years ago, it may be that it originally possessed the property of rodent infectivity, but lost it because of numerous monkey transfers. Another question which may be raised is whether strains which are known not to be related immunologically to the Brunhilde virus are likely to be related to the Lansing strain. It was this supposition which led to the successful passage of Wallingford virus in rodents. Following the same suggestion, an attempt was made to passage the Leon strain in cotton rats because of our finding that it was not related to the Brunhilde virus, by tests of vaccination-immunity. This strain was isolated in Los Angeles in 1937, by Dr. John F. Kessel, who kindly supplied our sample. We have failed to passage the Leon strain in cotton rats, and moreover have shown that it is immunologically unrelated to both the Brunhilde and Lansing viruses.[12] The Leon strain is therefore representative of a third distinct immunological group of poliomyelitis viruses. It will be of interest to know whether other representatives of this group will be identified and whether they also lack the property of rodent infectivity.

It is of interest that when known Lansing-like viruses are examined as a group, they appear to be widely distributed geographically, and can be isolated from both paralytic and non-paralytic cases. Since they further occur in nasopharynx and feces, as well as in the C.N.S., and since Lansing antibodies are widely distributed in human serum, there seems to be no reason to doubt at the present time, that these viruses are important in the epidemiology of poliomyelitis. It remains to be seen whether they are of less importance in this regard than viruses of the non-Lansing type.

Summary. The Wallingford virus, isolated from the nervous system of a fatal poliomyelitis case in California in 1934, has been successfully passaged in cotton rats and in mice. Material from each of 2 paralysed cotton rats produced typical poliomyelitis in one rhesus monkey. The virus has been tested for immunological relationships by inoculation in monkeys vaccinated with, and shown to be immune to, the Lansing and the Brunhilde viruses, respectively. These two viruses are

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[13] Bodian, D., Morgan, I. M., and Schwerdt, C. E., to be published.

representatives of 2 distinct types of poliomyelitis virus. The Wallingford virus is indistinguishable from the Lansing virus by the vaccination-immunity test, and unrelated to the Brunhilde virus by the same test.

A relationship between the property of rodent infectivity and the immunological specificity of the Lansing group of poliomyelitis viruses is suggested, in view of evidence for the existence of three distinct immunological groups of poliomyelitis viruses, only one of which has thus far been shown to be infective in rodents.

16806

Transmission of the Hamster-Adapted Newcastle Virus to Swiss Albino Mice.

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Following the adaptation of Newcastle virus, California Strain No. 11914, to the Syrian hamster,[1] attempts to transmit the modified virus of the 8th hamster passage to Swiss albino mice were successful through the fourth serial subinoculation only.[2] Brandly, *et al.*[3] working with egg propagated Newcastle virus reported similar results.

Further attempts to establish the infection in Swiss albino mice through the injection of hamster-adapted Newcastle virus of later hamster passages were likewise unsuccessful beyond several subinoculations.

Concentrated virus of the 203rd hamster passage was prepared from 8 infected hamster brains. The brain tissue was ground with alundum and diluted to a 10% suspension with physiological saline solution. Eighteen cc of the suspension were centrifugated under aseptic conditions at 50,000 RPM for 2 hours in a Spinco ultra-centrifuge. The supernate was discarded and the sediment was resuspended in 3 cc of physiological saline solution. Approximately .05 cc of the concentrated virus suspension was injected intracerebrally into each of 12 Swiss albino mice CFW.*

The use of this concentrated virus of the 203rd passage for the initial mouse inoculation established an infection in which slightly more than 10% of the injected mice of the first 8 passages showed symptoms of central nervous system involvement. From the 9th through the 20th passages more than 60% of the injected mice showed the characteristic symptoms. Table I shows the results of these 20 serial passages.

In mice responding to the virus injection irritability and malaise appeared in 2 to 9 days. In most cases these symptoms were followed by paralysis, evidenced first in the forelegs and followed in some cases by complete prostration within several hours. In other cases complete paralysis did not appear for as long as 24 hours after the forelegs became affected. Rhythmical jerking of groups of muscles or the entire body of paralyzed mice was frequently noted, accompanied by labored respirations. Death occurred within a few hours after the animals became moribund.

Brain material for passage was chosen from mice sacrificed when nervous symptoms developed. The virus-bearing brain material was ground with alundum and diluted to 10% suspension with physiological saline solution.

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[1] Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

[2] Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 427.

[3] Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. E., *Am. J. Vet. Res.*, 1946, **7**, 289.

* The Webster strain of Swiss albino mice from Carworth Farms, Rockland Co., N. Y., weighing between 10 and 12 g were used for all passages.

TABLE I.
Mouse Passage of a Strain of Hamster-Adapted Newcastle Virus.

Passage No.	No. animals inoculated*	Animals paralyzed moribund or dead No.	%	No. days after inoculation paralysis occurred
1	12	2	17	4-5
2	10	1	10	6
3	12	2	17	5-6
4	16	1	6	6
5	18	2	11	3-5
6	10	3	30	3-4-5
7	17	2	12	2-3
8	18	2	11	4-6
9	14	2	14	4-5
10	18	4	22	2-3-4-7
11	20	4	20	2-3-6-7
12	12	4	33	4-6-7-9
13	10	7	70	2-3-4
14	25	16	64	2-3-4
15	22	13	59	2-3
16	10	10	100	1
17	8	8	100	1-2
18	18	16	88	1-2-3
19	28	24	86	2-3-4-5
20	20	17	85	2-3-4-5

* Intracerebrally.

TABLE II.
Neutralization Test Results.

	Virus dilutions 10^{-1}	10^{-2}	10^{-3}
Specific chicken antiserum	0/4	0/4	0/5
Normal chicken serum	4/4	4/4	1/4
Virus titration, 15th mouse passage	7/7	4/4	2/4

Numerator of fraction denotes the number of deaths.
Denominator of fraction denotes number of mice inoculated.

Amounts of .03 to .05 cc of this suspension were injected intracerebrally throughout the 20 passages.

A virus neutralization test was conducted using brain suspension of the 15th mouse passage with specific Newcastle virus immune and normal chicken sera and with mice as the test animals. Specific immune serum completely neutralized the mouse brain virus whereas normal chicken serum had no effect. The virus titrated 10^{-3} according to the Reed-Muench calculation.[4] Details are given in Table II.

Infected mouse brain suspension of the 8th passage prepared as previously described was injected intracerebrally in 4 young Syrian hamsters. All hamsters showed symptoms characteristically seen in animals succumbing to similar injections of hamster adapted virus. The same results were observed in hamsters injected with mouse brain of the 18th mouse passage.

Summary. The hamster-adapted Newcastle virus (California strain Number 11914) of the 203rd passage has been successfully transmitted to Swiss albino mice and carried through 20 serial passages in this species by intracerebral inoculation. The virus produced symptoms of irritability and malaise usually followed by paralysis, and often accompanied by a characteristic nervous jerking with labored breathing. Mice showing typical symptoms of central nervous system involvement did not recover. Positive Newcastle chicken serum neutralized the virus from the 15th mouse passage while normal chicken serum failed to neutralize the virus. The virus of the 15th mouse passage titred 10^{-3} in mice by intracerebral inoculation. The mouse-adapted virus proved pathogenic for Syrian hamsters upon intracerebral injection.

The authors wish to express appreciation to Miss Dorothy M. Schenck for technical assistance.

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[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

16807

Histochemical Specificity of Phosphatases.*

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The question of the unity or plurality of phosphatases (Ph) has been a moot point for over 20 years, with numerous champions on both sides. There can be no doubt that alkaline and acid Ph are distinctly different enzymes; also the individuality of adenosinetriPh of muscle,[1] of pyroPh[2] and of hexosediPh[3] appears to be firmly established. However, a number of workers maintain that, besides these generally recognized differences, Phs differ among themselves in 3 more respects: 1 substrate specificity; 2, organ specificity and 3, specific activation and inhibition effects. The latter 2 groups often overlap.

As far as substrates specificity is concerned, Forrai[4] thinks that there is a specific sucrosePh and other sugarPhs; according to King,[5] lecithin is dephosphorylated by an enzyme not identical with the nonspecific Ph; Roche and Latreille[6] maintain that the kidney contains, besides glyceroPh, a phenylPh; Reis[7,8] claims the existence of a 5-nucleotidase; Bowers and coworkers[9] believe that aminoethylphosphate is hydrolyzed by a special enzyme; Waldschmidt-Leitz and Koehler,[10] Ichihara,[11] and Bredereck and Geyer[12] maintain that there is a special "phosphamidase" hydrolyzing the P-N bond.

There are data available to the effect that alkaline Phs of different organs may be actually different enzymes as shown by their slightly different resistance to inhibitors or by different pH optima. Belfanti and coworkers,[13] and Hommerberg[14] believe that bone Ph is different from renal or hepatic Ph. Bodansky[15] thinks that intestinal Ph can be distinguished from bone or renal enzyme. Masayama and Shuto[16] found a Ph in hepatomas, different from that of the normal liver.

Cloetens[17,18] distinguishes two alkaline Phs on the basis of their different degrees of activation by Mg. Drill, Annegers and Ivy[19] find that in jaundice a Ph appears in the plasma, different from the normal enzyme in respect to inactivation by cyanide.

Whether the differences reported should be considered as indications of the existence of several truly different enzymes, or as results of the admixture of various activators and inhibitors or of differences in technic cannot be decided on the basis of data available. The question is: Can histochemical technic be

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[1] Engelhardt, V. A., and Liubimowa, M. N., *Nature*, 1939, **144**, 668.

[2] Bamann, E., and Gall, H., *Biochem. Z.*, 1937, **293**, 1.

[3] Gomori, G., *J. Biol. Chem.*, 1942, **148**, 139.

[4] Forrai, E., *Biochem. Z.*, 1924, **145**, 54.

[5] King, E. J., *Biochem. J.*, 1931, **25**, 799.

[6] Roche, J., and Latreille, M., *Enzymologia*, 1937, **3**, 75.

[7] Reis, J., *Enzymologia*, 1937-38, **2**, 110.

[8] Reis, J., *Enzymologia*, 1938, **5**, 251.

[9] Bowers, R. V., Outhouse, E. L., and Forbes, J. C., *J. Biol. Chem.*, 1940, **132**, 675.

[10] Waldschmidt-Leitz, E., and Koehler, F., *Biochem. Z.*, 1933, **258**, 360.

[11] Ichihara, M., *J. Biochem.* (Japan), 1933, **18**, 87.

[12] Bredereck, H., and Geyer, E., *Z. physiol. Chem.*, 1938, **254**, 223.

[13] Belfanti, S., Contardi, A., and Ercoli, A., *Biochem. J.*, 1935, **29**, 842, 1491.

[14] Hommerberg, C., *Z. physiol. Chem.*, 1929, **185**, 123.

[15] Bodansky, O., *J. Biol. Chem.*, 1937, **118**, 341.

[16] Masayama, T., and Shuto, M., *Gann*, 1940, **34**, 176.

[17] Cloetens, R., *Enzymologia*, 1939, **6**, 46.

[18] Cloetens, R., *Enzymologia*, 1939, **7**, 157.

[19] Drill, V. A., Annegers, J. H., and Ivy, A. C., *J. Biol. Chem.*, 1944, **152**, 339.

utilized as an approach to the solution of the problem? A number of authors answer the question *in the affirmative*.[20-25]

Since many of the findings of the above mentioned authors could not be confirmed in this laboratory it was decided to reexamine the problem of the histochemical specificity of phosphatases on a broader basis.

Experimental. The preparation of tissue sections of mouse, rat, guinea pig, dog and human material was essentially the same as reported previously.[26] Six to 20 different tissues of material fixed in chilled alcohol or acetone were embedded in a single paraffin block. In view of the large molecular size of some of the substrates the slides were not coated with collodion. They were incubated for 1 to 24 hr.; phosphate precipitates were visualized by the sulfide technique. The following 19 substrates were used: Metaphosphate; pyrophosphate; methyl-, glycero- and aminoethylphosphate; phenyl, o-chlorophenyl, α-naphthyl-, resorcyl and phenolphthalein-phosphate; glucose-1-phosphate, hexosediphosphate, adenosinetriphosphate, yeast nucleate and thymonucleate; phosphorylcholine and lecithine;octanoylphosphate; p-chloranilidophosphonate. Phosphoric esters not available on the market were synthesized by esterification with $POCl_3$;[27] p-chloranilidophosphonate was prepared by the method of Otto;[28] octanoylphosphate was the gift of Dr. A. Lehninger of the Department of Biochemistry. The concentrations of the substrates ranged from 0.02 to 0.005M or, in the case of nucleates and lecithin, from 0.1 to 0.5%. Experiments were *performed at pH5* (*acetate buffer*), pH7 (tris(hydroxymethyl)-aminomethane-maleate buffer)[29] and pH9 (2-amino-2-methyl-1,3-propanediol buffer).[30] The cation used to trap the phosphate ions was Ca at pH9 and Pb at pH5 and 7. In most cases the concentration of Ca was 0.01M; that of Pb, 0.003M. In some cases, owing to special conditions of solubility, the concentration of the cation had to be changed. For instance, the highly insoluble meta- and pyrophosphates of Ca and Pb will go into solution in the presence of an excess of PO_3- or $P_2O_7\equiv$ ions. Therefore, a dilute solution of $CaCl_2$ or of $Pb(NO_3)_2$, respectively, was added drop by drop, with constant stirring, to the buffered substrate, until a slight permanent turbidity resulted. This was centrifuged off, and the clear supernatant was used. In other cases, as with phenyl-, o-chlorophenyl- and naphthylphosphate, phosphorylcholine, adenosinetriphosphate, nucleic acids and lecithin, the solubility of the Pb salts (in the case of lecithin and nucleic acids, even that of the Ca salts) proved to be so low that only a very small amount of cation was tolerated without precipitation. It is felt that some of the unsatisfactory results obtained with this latter group of substrates at pH5 and 7 were due to the very poor solubility of the Pb salts of the esters.

Some substrates (octanoylphosphate, adenosinetriphosphate, chloranilidophosphonate) have a slow rate of spontaneous hydrolysis under the conditions of the histochemical experiment. To avoid indiscriminate precipitation of phosphate in the tissues, the Coplin jars were supported in an inclined position, and the slides were placed in them with the sections facing downward. In this way the precipitate collected on the back surface of the slides.

Results. Pyrophosphate did not give positive results at any pH. With metaphosphate a minimal reaction was obtained on the sur-

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[20] Glick, D., and Fischer, E. E., *Arch. Biochem.*, 1946, **11**, 65.

[21] Dempsey, E. W., and Singer, M., *Endocrinology*, 1946, **38**, 270.

[22] Dempsey, E. W., and Deane, H. W., *J. Cell. and Comp. Physiol.*, 1946, **27**, 159.

[23] Dempsey, E. W., and Wislocki, G. B., *Am. J. Anat.*, 1947, **80**, 1.

[24] Emmel, V. M., *Anat. Rec.*, 1946, **96**, 423.

[25] Nickerson, W. J., Krugelis, E. J., and Andrésen, N., *Nature*, 1948, **162**, 192.

[26] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 4.

[27] King, E. J., and Nicholson, T. F., *Biochem. J.*, 1939, **33**, 1182.

[28] Otto, P., *Ber. deutsch. chem. Ges.*, 1895, **28**, 617.

[29] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 354.

[30] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 33.

face of intestinal villi at pH9, otherwise the results were negative. With both substrates spurious reactions consisting of random staining of various tissue elements were seen in all pH ranges. The non-enzymatic nature of this staining could be proven by its presence in inactivated sections, treated for 5 min. with Lugol's solution prior to incubation. Otherwise at pH9 the pictures obtained with all substrates were identical as far as the distribution of the enzyme in the tissues and the relative intensity of the staining at various sites is concerned. Occasionally slides incubated with nucleates for over 5h showed a somewhat more pronounced staining of nuclei than that seen with glycerophosphate as a substrate. However, this difference was neither constant nor conspicuous. Methylphosphate, p-chloranilidophosphonate and especially lecithin were hydrolyzed very much slower than the rest of the substrates; pictures obtained in 24h with the three substrates mentioned were comparable in intensity to those obtained in 1h with the rest. Since no reaction different from that seen with glycerophosphate was observed in the liver with hexosediphosphate or in the muscle with adenosinetriphosphate, it must be assumed that hexosediPh and adenosinetriPh do not survive the embedding procedure.

At pH5 the results were variable. Satisfactory and constant pictures were obtained only with glycerophosphate and resorcinolphosphate, and only after acetone fixation. Alcohol fixation often resulted in patchy, uneven staining. With all other substrates the intensity of the staining was very much lower and, in addition, the results were capricious, even consecutive serial sections showing differences in staining. In the case of nucleate or lecithin as substrates there was often a nonspecific impregnation of mucin, random groups of nuclei, nerve and muscle fibers. Otherwise, the distribution of the enzyme was identical with all substrates and in all tissues, as far as it could be judged from the often unsatisfactory slides. The only exception was p-chloranilidophosphonate. This substrate produced pictures entirely different from the rest.

At pH7, as could be expected, various combination pictures of the typical distribution of acid and alkaline Phs were obtained. The picture normally seen at pH5 showed up as a fairly strong component only when glycerophosphate or resorcinolphosphate were used as substrates, and only after acetone fixation. With the other substrates and/or alcohol fixation the distribution of the enzyme in all tissues was identical with that of alkaline Ph, although the intensity of the staining was much lower. Artifacts consisting of non-enzymatic impregnation of various structures were even more marked than at pH5. Otherwise the results were the same with all substrates. Specifically, the differences due to variation of the substrate, reported by Dempsey and Wislocki,[23] could not be confirmed in either human or guinea pig material.

Since there are reports on the specific inhibitory effects of taurocholate[15] and of cyanide,[24] a number of slides were incubated at pH9 in the presence of these substances, glycerophosphate being used as a substrate. Taurocholate in concentrations from 0.002 to 0.01M produced no visible effect in any of the tissues. Cyanide in concentration from 0.001 to 0.01M caused a marked inhibition of enzymatic activity in all organs. In some cases pictures comparable to those published by Emmel[24] were obtained; in other cases an intense staining persisted in the intestine in the presence of 0.01M NaCN while at the same time the reaction in the kidney was wiped out completely. All possible transitions between these two extremes could be observed in slides carrying a number of kidneys and pieces of small intestine from several animals.

Comment. The dependence of enzymatic behavior on the presence of various colloid substances is well known.[31,32] Even purified enzymes may show widely varying pH optima with different substrates.[33] Owing to these and other factors, the decision on the identity or nonidentity of enzymes may be an exceed-

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[31] Rona, P., and Gyotoku, K., *Biochem. Z.*, 1926, **167**, 171.

[32] Mendel, B., and Rudney, H., *Science*, 1944, **100**, 499.

[33] King, E. J., and Delory, G. E., *Biochem. J.*, 1939, **33**, 1185.

TABLE I.

Comparison of Results Obtained with Various Substrates.

Six to 20 different tissues of humans, dogs, rats, mice, and guinea pigs mounted on each slide. Pattern of distribution of enzymatic activity in any given organ is constant and independent of the substrate used. All-over intensity of reaction denoted by plus signs. In the column of pH7, *5* indicates the normal distribution as seen at pH5, *9* that seen at pH9, glycerophosphate being used as a substrate. ? stands for unsatisfactory results.

Substrate	pH5	pH9	pH7
Glycerophosphate	+++	+++	*9* + to ++; *5* + to ++
Pyrophosphate.	—	—	—
Metaphosphate	—	—	—
Methylphosphate	—	+	—
Aminoethylphosphate	—	++ to +++	— to *9* +
Phenylphosphate o-Chlorophenylphosphate α-Naphthylphosphate Phenolphthaleinphosphate	— to + ?	++ to +++	*9* + ?
Resorcylphosphate	+++	+++	*9* + to ++; *5* + to ++
Glucose-1-phosphate	—	+++	—
Hexosediphosphate	— to + ?	+++	— to *9* +
Adenosinetriphosphate	—	+++	—
Yeast nucleate	+ to ++	++ to +++	*9* + to ++; *5* +
Thymonucleate	— to + ?	++	*9* +
Phosphorylcholine	—	++	not done
Lecithine	—	+	,, ,,
Octanoylphosphate	not done	++	,, ,,
p-Chloranilidophosphonate	special	+	*9* +

ingly difficult one, even if quantitative chemical procedures are used. The histochemical approach to the problem is beset with the danger of additional sources of error among which only a few will be mentioned.

1. The two main factors regulating the deposition of poorly soluble phosphates in tissue sections are the rates of phosphate production and of diffusion. Varying ratios between these two factors may result in an "all or none" effect, depending on whether the rate of phosphate production will or will not overtake that of diffusion to a point where the solubility product of the phosphate in question is exceeded locally. Any kind of inhibition of enzymatic action (partial inactivation of the enzyme by unsuitable fixation; non-optimal pH or substrate; the presence of various inhibitors) is likely to obliterate areas below a critical level of activity, while areas of much higher activity may remain apparently uninfluenced or only slightly weakened.

2. In the case of substrates of large molecular size the different rates of diffusion into various tissue elements may be a decisive factor.

3. The nonenzymatic impregnation of tissue structures by Pb is well known.[34] The danger of such impregnation is especially great when, on account of long periods of incubation, spontaneous hydrolysis of the substrate may lead to supersaturation in respect to Pb (possibly also of Ca) phosphate. Such artifacts may be recognized by *simultaneously incubating inactivated tissues as controls.*

Since some of the complications of the histochemical technic cannot be avoided, great caution is warranted in the evaluation of its results, especially when non-optimal conditions are used. Only marked and uniform differences in the picture of enzymatic distribution, obtained by changing conditions such as substrate, pH or inhibitors and activators, can be accepted as a presumptive evidence for the presence of different enzymes. Results of this type have been reported in the case of acid Ph,[35] of acid adenosinetriPh of grain,[20] and of yeast Phs.[25] The importance of such minor differences as reported by Dempsey and Singer,[21] Dempsey and Deane,[22] Dempsey and Wislocki[23] and by Emmel[24] is questionable.

Summary. Nineteen different substrates were used in this study on the histochemical specificity of phosphatases. With 18 substrates no indication of the presence in paraffin-embedded mammalian tissues of phosphatases other than the common non-specific alkaline and acid variety was found. With one of the substrates, p-chloranilidophosphonate, a strikingly different picture of enzymatic distribution was observed in the acid range, but otherwise the pattern of distribution of enzymatic activity in any given organ was constant and independent of the substrate used.

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[34] Lassek, A. M., *Stain Techn.*, 1947, **22**, 133.

[35] Gomori, G., *Arch. Path.*, 1941, **32**, 189.

16808

Effect of Tetraethylammonium on Venous and Arterial Pressure in Congestive Heart Failure.

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That widespread vasoconstriction occurs during congestive failure was first proposed many years ago by Starling[1] and by Bolton.[2] Recently, it has been suggested that reflex vasoconstriction contributes to the maintenance of the usually normal arterial pressure[3,4] and the elevated venous pressure[3,5,6] in heart failure. Conversely, the fall in calculated peripheral resistance accompanying the improved output of the digitalized failing heart[7,8] speaks for a release of vasoconstriction. Renin has been found in the renal veins of patients in congestive failure,[3] and the reduction of hepatic and renal blood flows in such cases is further evidence of increased peripheral resistance attributable to vasoconstriction.

Tetraethylammonium chloride is a quaternary ammonium salt which transiently blocks the transmission of impulses through peripheral autonomic ganglia.[9] Hayward[10] has recently observed that tetraethylammonium bromide decreases the venous and arterial pressures in hypertensive patients with compensated or failing circulations. Since peripheral resistance is greatly increased in essential hypertension, it was of interest to observe the effect of this drug on the venous and arterial pressures of normotensive patients with and without failure, in an attempt to delineate the role of vasoconstriction in congestive failure *per se.*

Methods. Venous pressure was measured directly with a No. 18 gauge needle in an antecubital vein, using the sternal angle of the supine patient as a reference point. Arterial blood pressure was measured with a sphygmomanometer and the mean pressure was arbitrarily taken to be the average of the systolic and diastolic pressures. The heart rate was counted at the apex for 30 seconds. After repeated observations of heart rate and pressures had established a constant baseline, tetraethylammonium chloride (TEAC)* in doses of 2 to 6 mg/kg of body weight was injected through a fresh venipuncture over a period of 2 to 4 minutes, and the pertinent observations were continued frequently for 35 to 40 minutes thereafter.

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[1] Starling, E. H., The Fluids of the Body. W. T. Keener and Co., Chicago, 1909.

[2] Bolton, C., *Brit. Med. J.*, 1917, **1**, 642.

[3] Merrill, A. J., Morrison, J. L., and Brannon, E. S., *Am. J. Med.*, 1946, **1**, 468.

[4] Landis, E. M., Brown, E., Fauteux, M., and Wise, C., *J. Clin. Invest.*, 1946, **25**, 237.

[5] Starr, I., and Rawson, A. J., *Am. J. Med. Sci.*, 1940, **199**, 27.

[6] Warren, J. V., and Stead, E. A., Jr., *Arch. Int. Med.*, 1944, **73**, 138.

[7] Stead, E. A., Jr., Warren, J. V., and Brannon, E. S., *Arch. Int. Med.*, 1948, **81**, 282.

[8] Bloomfield, R. A., Rapaport, B., Milnor, J. P., Long, W. K., Mebane, J., and Ellis, L. B., *J. Clin. Invest.*, 1948, **27**, 588.

[9] Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, **87**, 220.

[10] Hayward, G. W., *Lancet*, 1948, **1**, 18.

* Etamon Chloride, Parke-Davis and Company.

Twenty subjects were studied in the manner described. Group I contained 8 patients with normal arterial and venous pressures, without evidence of cardiovascular disease. Group II included 2 compensated normotensive cardiacs with normal venous pressures and circulation times. Group III consisted of 8 patients with congestive heart failure as evidenced by distended neck veins, basilar rales, enlarged liver, and (except for patient G.H.) edema. None of these patients had a diastolic blood pressure above 90, and all of them, except G.H., were taking digitalis at the time of the study. Group IV consisted of 2 other patients with congestive failure in whom responses were compared before and after effective treatment for congestive failure. One of these patients (W.G.) had slight diastolic hypertension.

Results. The results are summarized in Table I.

Groups I and II. Venous pressures in the normal subjects and compensated cardiacs did not change, or rose slightly. Results were similar when the pressure in the antecubital vein was artificially elevated to levels seen in congestive failure by lowering the arm. Mean arterial blood pressure fell an average of 2.8 mm Hg ± 10.9 mm and the pulse rate always increased. The results were the same in normal subjects placed in a semi-reclining position 60 degrees from the horizontal.

Group III. Following injection of TEAC, venous pressure of patients in congestive failure promptly dropped 35 to 80 mm below the baseline. The effect began one to 2 minutes after injection was started, when 100 to 200 mg of the drug had entered the circulation, and was maximal in 5 to 7 minutes. The pressure then started to rise slowly, but usually had not reached its pre-injection level at the end of half an hour. Although there was some overlapping, the average drop in mean arterial blood pressure (24.6 mm Hg ± 5.6 mm) in Group III was significantly greater than the change seen in Groups I and II ("t" equals 4.25; "P" equals < 0.01). In contrast to the normals, 5 of the 8 patients in Group III had no change in heart rate, 2 slowed slightly, and only one (E.B.) developed a tachycardia. In this patient, a young man with calcified constrictive pericarditis, a tachycardia of the same degree was produced by 1 mg of atropine intravenously, without any change in venous pressure.

Group IV. Two patients in congestive failure were studied before and after digitalization. Before treatment, the injection of TEAC was followed by a sharp fall in venous pressure and a drop in mean arterial pressure. Several days later, when both patients had recovered considerably, the experiment was repeated. In W.G., who had become free of congestive signs and symptoms, TEAC caused no fall in venous pressure. In patient A. H., who still had dyspnea on exertion and basal pulmonary rales, TEAC caused a slight fall in venous pressure. In both patients, the percentile drop in mean arterial pressure was equivalent to that before digitalization.

Arm-to-tongue circulation times, measured with Decholin in 5 subjects before and immediately after injection of TEAC, did not change consistently. No untoward reactions to TEAC were observed, and no change in the degree of the cardiac patients' dyspnea was apparent.

Discussion. In high concentrations, tetraethylammonium increases the cardiac output in a failing heart-lung preparation.[11] However, the drug does not increase the output in dogs under barbiturate anesthesia, and it increases the human cardiac output only slightly or not at all.[12] Moe believes that any increases in output are probably attributable to the tachycardia.

Because the action of TEAC is chiefly on the peripheral autonomic ganglia, the data here presented imply the existence of an increased autonomic vascular tone in congestive failure which contributes to the elevation of venous pressure as well as the maintenance of arterial pressure. Almost the entire peripheral

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[11] Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1945, **84**, 189.

[12] Moe, G. K., personal communication.

TABLE I.

Pt., age and sex	Diagnosis*	Dose TEAC, mg/kg	Venous pressure, mm 5% glucose		Arterial pressure, mm Hg.			Heart rate, per min.	
			Baseline corrected to sternal angle	Max. change	Baseline B.P.	Mean B.P.	Max. change in mean B.P.	Baseline	Max. change
Group I									
F.E. 24 M	Normal	6	300†	0	120/72	96	+ 3	74	+ 6
G.G. 25 M	,,	6	0	0	128/83	106	0	70	+36
D.F. 24 M	,,	6	0	+ 10	110/75	93	— 8	80	+32
M.W. 18 F	Latent syphilis	4	80	0	130/82	106	—16	100	+12
D.B. 48 M	Peptic ulcer	6	78	+ 10	142/84	113	— 5	80	+16
V.C. 29 F	Hemorrhoids	6	135	+ 10	116/76	96	— 4	112	+32
A.Mc. 31 M	Early syphilis	6	30	+ 10	118/72	95	+16	62	+52
O.B.‡ 21 M	,, ,,	6	10	+ 5	158/60	112	0	80	+60
Group II									
M.C. 18 M	RHD, MS, AS, AI	6	35	0	120/36	78	+ 7	76	+24
G.F. 75 M	ASHD, AF	6	95	+ 15	148/64	106	—21	74	+14
		Mean:	51.5 ± 38.8	+6.6 ± 5.8		100.1 ± 10.4	—2.8 ± 10.9		
Group III									
G.H. 54 M	ASHD, My	2	110	— 55	96/60	78	—10	98	— 8
E.B. 24 M	CP, AF	3	165	— 65	110/80	95	—21	84	+22
J.Y. 58 M	CP, AF	6	200	— 70	126/68	97	—27	74	0
W.H. 64 M	SA, AI	4	25	— 35	180/87	134	—19	80	0
M.M. 61 M	RHD, MS, MI, AI, TI, AF	6	80	— 80	128/65	97	—25	62	0
J.W. 49 M	ASHD, AF, ?RHD, ?TI	6	195	— 75	100/76	88	—18	90	0
A.E. 47 M	RHD, MS, AS, AI, AF	6	80	— 45	170/60	115	—31	68	—10
J.D. 65 M	ASHD, ?CP	6	65	— 70	110/78	94	—46	92	0
		Mean:	115 ± 64.5	—61.9 ± 15.6		99.8 ± 17.2	—24.6 ± 10.7		
Group IV									
W.G. 31 M	HHD, ASHD (before digitalis)	3.6	240	—120	146/120	133	—29	130	+36
W.G.	(After digitalis)	4.2	60	+ 25	130/96	113	—21	98	0
A.H. 66 M	ASHD (before digitalis)	4.5	155	— 90	134/82	108	—60	112	0
A.H.	(After digitalis)	5.4	—25	— 20	118/80	99	—53	108	0

* RHD—Rheumatic Heart Disease. ASHD—Arteriosclerotic Heart Disease. HHD—Hypertensive Heart Disease. SA—Syphilitic Aortitis. MS—Mitral Stenosis. AS—Aortic Stenosis. AI—Aortic Insufficiency. TI—Tricuspid Insufficiency. CP—Constrictive Pericarditis. My—Myocardial Infarction.

† The arm was lowered far below the level of the heart, but no correction to the level of the sternal angle was made. This value is not included in the calculation of the mean.

‡ This patient had received 0.5 mg of adrenalin subcutaneously 40 minutes before these observations.

vascular bed is under active vasomotor control.[13] A reduction in the amount of blood pumped from the venous to the arterial side of the circulation would lower arterial pressure while tending to elevate venous pressure.[1,2,5] It seems possible, therefore, that whenever cardiac output is inadequate, reflex peripheral autonomic mechanisms are called into play. These would maintain arterial pressure by arteriolar constriction and tend to increase cardiac output by further increasing the venous filling pressure. Venous filling pressure might be elevated by an increased tone in the large veins or by a redistribution of blood resulting from constriction of smaller peripheral vessels. These experiments do not exclude the possibility that other factors such as increased blood volume or tissue fluid pressure may also play major roles in the maintenance of the elevated venous pressure of congestive failure.

Finally, it should be pointed out that only the peripheral venous pressure was measured in these experiments. It is possible, particularly in subjects without venous hypertension, that pressure changes in the central veins and right atrium were not closely reflected by changes in the peripheral venous pressure.

Summary. Tetraethylammonium chloride given intravenously to 9 normotensive patients in congestive failure caused a precipitous fall in venous pressure and a significant decrease in arterial pressure in every case. Compensated cardiacs and non-cardiac normotensive controls showed no fall in venous pressure and a much smaller change in arterial pressure. Following digitalization, 2 cardiac subjects in failure lost some of their initial responsiveness to TEAC.

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[13] McDowall, R. J. S., *Phys. Rev.*, 1935, 15, 98.

16809

Survival of Rats After Temporary Complete Renal Ischemia.*

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It has been shown that rats generally survive bilateral interruption of the renal circulation for periods lasting up to one hour.[1] With longer intervals of complete ischemia, the mortality rises sharply. Cessation of blood flow to both kidneys for two hours is uniformly fatal; there is progressive elevation of BUN and death occurs in uremia within a few days.

The principal lesion produced by temporary periods of complete renal ischemia is necrosis of the proximal convoluted tubules.[1] In rats with a one hour period of bilateral ischemia, the necrosis is followed by repair and survival of the animals. Rats with a 2 hour period of ischemia evidently die in uremia before regeneration is effective.

In order to determine whether the renal lesion produced by periods of ischemia longer than those in the preceding experiments was subject to repair and would permit survival of the rats, unilateral ischemia was produced. The left main renal artery and vein of 50 adult white rats were occluded with a bulldog clamp for periods ranging from 2 to 4 hours. A 5 hour period was unsatisfactory because of the frequent occurrence of renal infarction. Heparin was given intravenously before clamping, and in some instances during clamping, to retard intrarenal thrombosis. The right kidney was intact. The animals were sacrificed at various intervals up to 5 months after removal of the clamps.

Results. Tubular necrosis was followed by

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* Aided by a grant from the Elisabeth Severance Prentiss Foundation.

[1] Koletsky, Simon and Gustafson, G. E., *J. Clin. Invest.*, 1947, 26, 1072.

TABLE I.
Mortality in 75 Rats After Complete Ischemia of Left Kidney and Subsequent Resection of the Right Kidney.

Duration of left renal ischemia, hrs	No. of rats	No. of rats which died	Mortality, %
1½	15	0	0
2	15	1	7
3	15	6	40
3½	15	9	60
4	15	13	87

repair in all rats. At the end of one week the tubules were largely cleared of necrotic débris and were lined by a newly formed epithelium. However after one week there was progressive and profound renal atrophy so that at 3 weeks the kidney, although completely repaired, was reduced to approximately one half to one third the normal size. Both glomeruli and tubules were atrophic and the interstitial fibrous stroma increased in amount. The tubular lumens were narrow or closed and the lining cells small. Further observations showed that the atrophy resulting from temporary ischemia persisted indefinitely as long as the complete opposite kidney was intact.

The functional capacity of the small atrophic kidney resulting from temporary interruption of blood flow was then investigated. Clamps were applied to the left main renal artery and vein of 75 adult rats divided into 5 groups of 15 rats each, with the following periods of occlusion respectively, *i.e.*, 90 minutes and 2, 3, 3½, and 4 hours. The right kidney was resected 3 weeks after removal of the clamp. BUN was determined by the method of Ormsby[2] before and after release of the clamps and at regular intervals following resection of the right kidney. Heart's blood was obtained for BUN in rats which died in uremia. Surviving animals were sacrificed from 3 to 5 months after the right kidney was removed.

Results. The mortality among the 5 groups of rats is shown in Table I. In the rats which died the BUN usually rose rapidly and death occurred within 1 or 2 weeks after resection of the right kidney. Terminal BUN values ranged from 220 to 764. Four animals developed chronic uremia with BUN levels up to 300 and marked weight loss, and these died from 4 to 11 weeks after resection of the right kidney.

All rats in the 90 minute group and 14 of 15 in the 2 hour group survived. In the 3 and 3½ hour groups, 9 and 6 animals survived respectively as compared to only 2 survivals in the 4 hour group. All surviving rats were in good condition and either maintained or gained weight. However, except for most animals in the 90 minute and 2 hour group, the BUN subsequent to resection of the right kidney was permanently elevated. The levels were usually below 100 mg per 100 cc with ischemia up to 3 hours' duration and above 100 in the animals with 3½ and 4 hour periods of ischemia. Two of the 6 surviving rats with 3½ hour renal ischemia, after being in good condition for 4 months, had a sudden rise in BUN and developed uremia. At autopsy the kidneys were the seat of necrotizing arteriolitis, especially the glomeruli, and also acute pyelonephritis.

In all surviving rats resection of the right kidney was followed by marked compensatory hypertrophy of the left kidney. The latter was large, usually exceeding normal size, and both glomeruli and tubules were hypertrophic. In contrast the rats which died following removal of the right kidney had a small atrophic left kidney with minimal or no compensatory hypertrophy.

Conclusion. These experiments indicate that if the period of complete ischemia does not exceed 2 hours, the rat kidney can regain enough function to permit survival. With complete ischemia of 3 hours' duration chance of survival is reduced to about half. Cessation of renal blood flow for more than 3 hours usually results in loss of capacity for compensatory hypertrophy and hence may be considered irreversible.

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[2] Ormsby, A. A., *J. Biol. Chem.*, 1942, **146**, 595.

16810

Electroencephalograms in Behavior Changes in Cats.

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Lesions of the ventromedial hypothalamic nuclei in cats usually are accompanied by striking behavior changes, depending upon the symmetry and completeness of these rather small lesions (Wheatley[1]). Postoperatively these animals display a malevolent attitude, are extremely difficult to handle and thereafter are quite untameable. Hyperphagia is frequently part of this syndrome and the animals may become very obese. There are no motor defects and their behavior is well-controlled and purposeful, not to be compared with the sham rage of decorticate preparations. How these small, subcortical lesions produce such effects has not been explained, although a disturbance in hypothalamo-cortical relationships is presumably involved. Since clinical observations[2-11] have indicated that a high proportion of patients showing behavior disorders may have abnormal electroencephalograms, and since Hoagland[2] observed that emotional stimulation may increase delta activity from both hypothalamus and cortex, it was thought that electroencephalographic observations on these cats might indicate some physiological change in cortical activity.

The bilateral lesions were produced by electrolysis, and in all cases of savage behavior were found to be restricted to the immediate vicinity of the ventromedial nuclei. All cats used were in good health at the time of experiment. The experiments were performed as follows: Fifteen mg of beta erythroidin hydrobromide in 0.9% NaCl solution were injected intravenously, and an intratracheal catheter inserted. Artificial respiration was maintained by positive pressure, the respirator being set to provide 27 inspirations a minute. Complete paralysis was maintained by the administration of a solution of erythroidin containing 0.5 mg per cc, given intravenously at a suitable rate. Our experience, in agreement with that of others,[12] indicates that such curarization does not significantly affect the EEG. Silver electrodes were fixed to the scalp with electrode paste and collodion. The numerical designation and position of the leads were: 1, occipital; 2, parietal (vertex); 3, frontal; 4, the common lead, was from the ear. Some of the resistance offered by skull, skin, etc., was eliminated by removal of bone from the skull areas beneath the electrode sites a week or more before the first run. Each animal was subjected to several twenty minute periods of recording. A six channel Offner electroencephalograph with a crystograph recorder was used.

Observations. Records from over a hundred experiments of this type are in general agreement with those of Clark and Ward[13] on normal cats with implanted leads and with similar preparations of our own. Normal

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[1] Wheatley, M. D., *Arch. Neurol. and Psychiat.*, 1944, **52**, 296.

[2] Hoagland, H., *J. Gen. Psychol.*, 1938, **19**, 227.

[3] Jasper, H. H., Solomon, P., and Bradley, C., *Am. J. Psych.*, 1938, **95**, 641.

[4] Lindsley, D. B., Cutts, K. K., *Arch. Neurol. and Psychiat.*, 1940, **44**, 1199.

[5] Knott, J. R., and Gottlieb, J. S., *Arch. Neurol. and Psychiat.*, 1944, **52**, 515.

[6] Gottlieb, J. S., Knott, J. R., and Ashby, M. C., *Arch. Neurol. and Psychiat.*, 1945, **53**, 138.

[7] Michaels, J. J., and Secunda, L., *Am. J. Psychiat.*, 1944, **101**, 407.

[8] Michaels, J. J., *Psychosomatic Medicine*, 1945, **7**, 41.

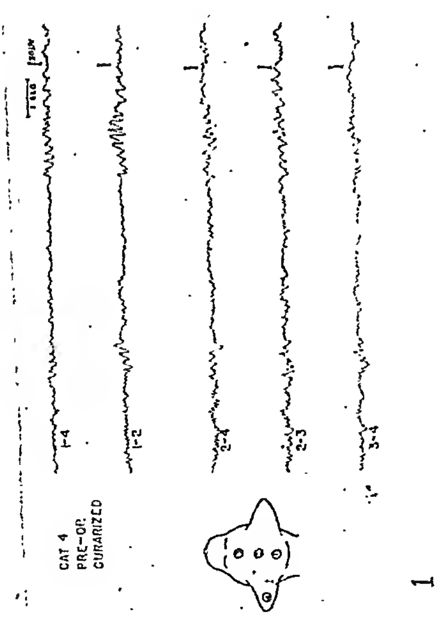
[9] Will, O. A., *U. S. Naval Med. Bull.*, 1945, **44**, 341.

[10] Simon, D. J., and Diethelm, O., *Arch. Neurol. and Psychiat.*, 1946, **55**, 619.

[11] Rockwell, F. V., and Simons, D. J., *Arch. Neurol. and Psychiat.*, 1947, **57**, 71.

[12] Girden, E., *J. Neurophysiol.*, 1948, **11**, 169.

[13] Clark, S. L., and Ward, J. W., *J. Neurophysiol.*, 1945, **8**, 98.



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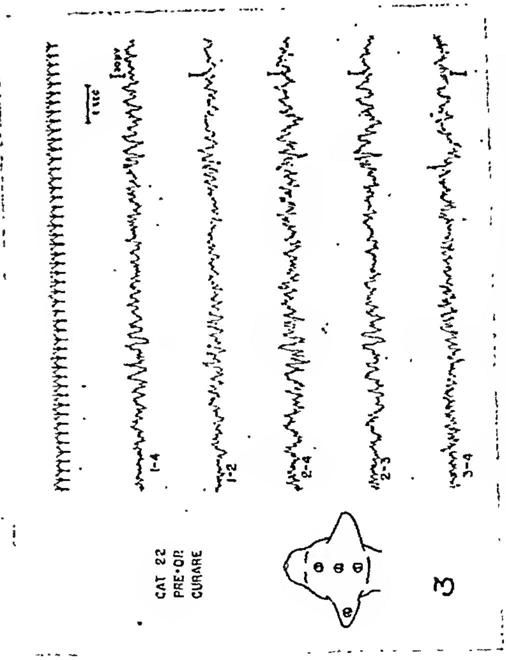

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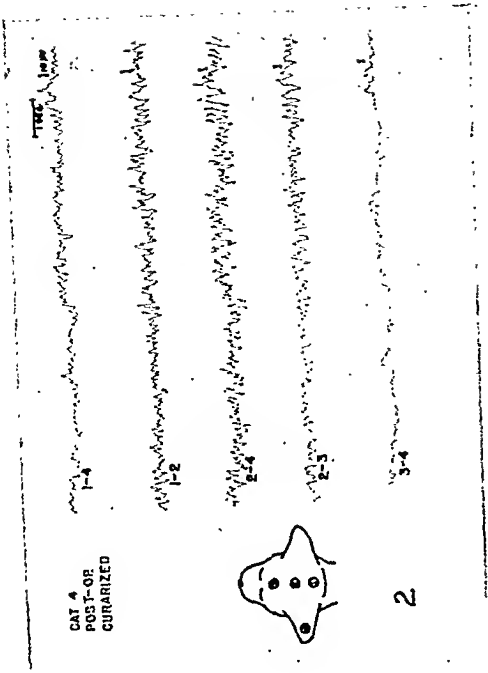

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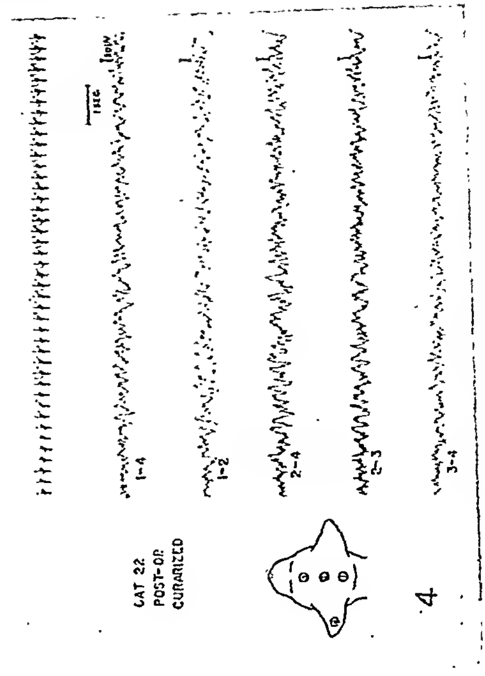

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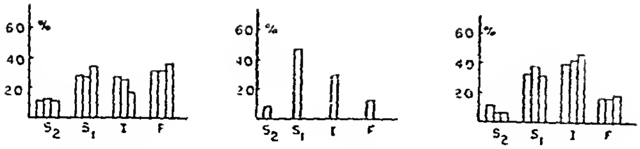
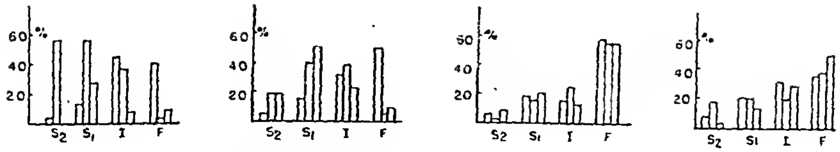
FIG. 1. Preoperative EEG of cat 4, which later became savage.
FIG. 3. Preoperative EEG of cat 22, a control.

FIG. 2. Postoperative EEG of cat 4, in savage state.
FIG. 4. Postoperative EEG of cat 22, a control.

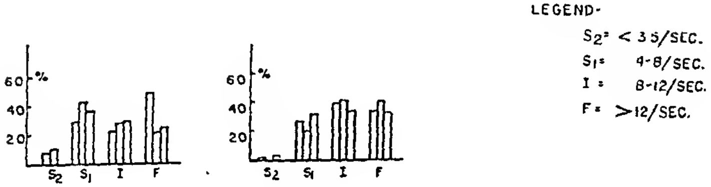
cats show a striking variability in the EEG as to frequency, amplitude and the occurrence of random bursts of fairly uniform frequencies, even when curarized. Differences between normal curarized and non-curarized animals are relatively slight. It is of some interest that EEG patterns resembling those which accompany the appearance of sleep in cats may occur with great frequency under erythroidin. Such patterns may be seen in



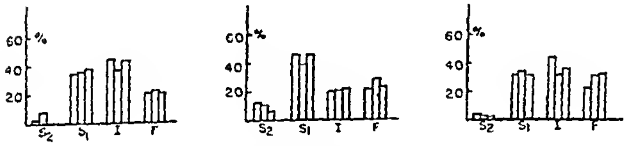
CAT AT. 2 PRE-OP



CAT AT.2 POST-OP. SAVAGE.



CAT 22 PRE-OP.



CAT 22 POST-OP.

FIG. 5.

Graphic representation of frequency analyses for cat 2, savage, and cat 22, control. Each graph represents a 20-minute run. Each vertical bar indicates the percentage of a frequency band in a 30-second sample of the record.

Fig. 1 and 2, especially from the occipital and vertex leads, but less evident from the frontal monopolar lead. Sample records of normal unoperated cats are shown in Fig. 1 and 3. Fig. 2 is a record from the same cat as in Fig. 1, after the animal had become extremely savage following operation for production of hypothalamic lesions. Fig. 4 is a postoperative record from the same cat as in Fig. 3, but in this case no savageness was present.

Inspection of records from normal and savage cats and comparison of preoperative and postoperative records from savage cats disclosed no qualitative differences which were considered to be outside the normal variation. In an attempt at more precise analysis, several 30 second samples from the vertex lead of each run were measured for frequency distribution. Four frequency categories were used: (a) very slow (S_2), less than 3.5/sec.; (b) slow (S_1), 4-8/sec.; (c) intermediate (I), 8-12/sec.; (d) fast (F), more than 12/sec. Examples of the distribution patterns in 2 cats, one savage and one unoperated control are shown in Fig. 5, and a summary of the findings in normal, savage and control cats will be found in Table I. Inspection of these data leads to the conclusion that no significant difference exists. This conclusion is strengthened by the degree of variability in the same animals, pre- and postoperatively, and justifies rejection of the hypothesis that any sig-

TABLE I.
Frequency Analysis of Normal and Operated Cats.
(Lead 2-4.)

	Avg % frequency			
Experiments	S_2	S_1	I	F
29 normal (13 cats)	8.86 ± 1.98*	37.48 ± 2.36	26.18 ± 2.2	27.13 ± 2.79
14 operated controls (5 cats)	3.62 ± 0.84	34.62 ± 4.33	38.5 ± 2.76	23.34 ± 4.64
19 malevolent (5 cats)	9.43 ± 1.64	32.06 ± 2.3	29.27 ± 1.79	30.66 ± 2.74

S_2 indicates frequencies less than 3.5 per second; S_1 indicates frequencies of 4-8 per second; I indicates frequencies of 8-12 per second; F indicates frequencies greater than 12 per second.
* Standard error.

nificant trends exist after this type of hypothalamic injury.

Corticohypothalamic relationships remain physiologically and anatomically obscure. Technical factors have interfered with anatomical studies (Ingram[14]). Neuronographic studies (Ward and McCulloch[15]; Murphy and Gellhorn[16]) indicate that connections between the frontal lobe, at least, and hypothalamus are fairly extensive. The significance of these connections is not clear. According to Kennard[17] and Obrador,[18] extensive destruction of the hypothalamus suppresses cortical potentials. On the other hand, Morrison, *et al.*[19] found no relationship between hypothalamic and cortical activity, and Jasper and Droogleever-Fortuyn[20] found no generalized modification of cortical potentials upon stimulation of the hypothalamus. Laufer[21] and Lennox and Brody[22] have reported that hypothalamic lesions in human patients are associated with generalized low frequency activity. Our own results indicate that relatively small, bilateral lesions restricted to the ventromedial part of the tuber do not alter cortical activity in curarized animals, even when such lesions may be considered causally related to decided changes in behavior with outward signs of an emotional type. However, records from the cortices of such animals under more nearly "normal" circumstances, unaffected by drugs, both at rest and when displaying their characteristic rage, have not yet been secured, and such data will be an essential part of the final interpretative picture. Under aggravation, savage cats show violent autonomic discharges, which are not detectable under erythroidin. Under non-narcotized conditions Darrow's[23] theory that such discharges may be associated with changes in cortical activity may then well be put to test.

Summary. Electroencephalographic studies of cats curarized with erythroidin show no discernible differences between cortical potential patterns in normals, operated controls and cats showing "savage" behavior after production of relatively specific hypothalamic lesions.

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[14] Ingram, W. R., *Res. Publ. Ass. nerv. ment. Dis.*, 1940, **20**, 195.

[15] Ward, A. A., and McCulloch, W. S., *J. Neurophysiol.*, 1947, **10**, 310.

[16] Murphy, J. P., and Gellhorn, E., *J. Neurophysiol.*, 1945, **8**, 431.

[17] Kennard, M. A., *J. Neurophysiol.*, 1943, **6**, 233.

[18] Obrador Alcalde, S., *J. Neurophysiol.*, 1943, **6**, 81.

[19] Morison, R. S., Finley, K. H., and Lothrop, G. N., *J. Neurophysiol.*, 1943, **6**, 243.

[20] Jasper, H. H., and Droogleever-Fortuyn, J., *Res. Publ. Ass. nerv. ment. Dis.*, 1947, **26**, 272.

[21] Laufer, M. W., *J. Nerv. and Ment. Dis.*, 1947, **106**, 527.

[22] Lennox, M., and Brody, B. S., *J. Nerv. and Ment. Dis.*, 1946, **104**, 237.

[23] Darrow, C. W., *Am. J. Psychiat.*, 1945, **102**, 791.

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Studies on the Blood Pyridoxine of Vitamin B_6 Deficient Monkeys.*

LOUIS D. GREENBERG AND JAMES F. RINEHART.

From the Division of Pathology, University of California Medical School, San Francisco, Calif.

Past experience has shown that in general the vitamin content of the blood is influenced by the quantity in the diet. In the case of vitamin C, thiamin and pantothenic acid, it is possible to use the blood level as a criterion of the adequacy of the intake. When this work was commenced, no reports on the concentration of the pyridoxine in blood of any species were to be found in the literature. Since then Ritchey *et al.*[1] using an assay with Neurospora have reported a value of 42 μg per 100 ml for pooled mouse blood. Subsequently Ray and co-workers[2] using a modification of the yeast method of Atkins and associates,[3] presented data on the blood pyridoxine content of control and cobalt deficient sheep. Employing a modification of the yeast method of Atkins and associates, our laboratory has carried out a rather extensive study of the blood pyridoxine in monkeys and humans and has studied its distribution in plasma and erythrocytes.

The present paper deals with the blood pyridoxine levels in monkeys and its alteration during deficiency of this vitamin. To date observations have been made on 14 deficient monkeys. A few of our early results were presented along with a description of the early manifestations of pyridoxine deficiency at the Federation meetings in 1946.[4] Since then our study has been greatly extended.

Experimental. Young rhesus monkeys weighing between 1.8 and 3.0 kg were used in these experiments. The methods employed in handling and feeding the animals have been described in an earlier publication.[5] The basal diet was a modified M-3 diet[6] and contained powdered sucrose 73, vitamin-free casein (GBI or Labco) 18, Hawk and Oser salt mixture 4, and corn oil 2. It was compressed into 2 g tablets on a Stokes tablet machine following granulation and the addition of 1% calcium stearate as a lubricant. The tablets were fed *ad libitum*. A vitamin tablet containing the following was fed daily; Nicotinic acid 5 mg, riboflavin 1 mg, thiamin chloride 0.5 mg, calcium pantothenate 3 mg, choline dihydrogen citrate 100 mg, para-aminobenzoic acid 100 mg, inositol 100 mg, ascorbic acid 25 mg, plus sufficient powdered sugar to make a tablet weighing 1.5 g. In addition the monkeys received by mouth twice weekly 5 drops vitamin A and D concentrate (100,000 I. U. vitamin A and 10,000 I. U. vitamin D per g), 385 μg† pteroylglutamic acid, 10 micrograms biotin and 5 drops of mixed natural tocopherols (Nopco) once a week. Control monkeys were also provided with 3.5 mg of pyridoxine hydrochloride twice a week.

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* This investigation was aided by grants from the California Fruit Growers Exchange and the Christine Breon Fund for Medical Research. We are grateful to Merck and Co., Rahway, N. J., for supplies of biotin and inositol; to Lederle Laboratories, Pearl River, N. Y., for pteroylglutamic acid; and to Mr. Stephen Dean of the College of Pharmacy for assistance in the preparation of diet tablets.

[1] Ritchey, M. G., Wieks, L. F., and Tatum, E. L., *J. Biol. Chem.*, 1947, **171**, 51.

[2] Ray, S. N., Weir, W. C., Pope, A. L., and Phillips, P. H., *J. Nutrition*, 1947, **34**, 595.

[3] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Indust. and Engr. Chem. Anal. Ed.*, 1943, **15**, 141.

[4] Greenberg, L. D., and Rinehart, J. F., *Fed. Proc.*, 1946, **5**, 222.

[5] Rinehart, J. F., Greenberg, L. D., and Ginzton, L. L., *Blood*, 1948, in press.

[6] Waisman, H. A., and McCall, K. B., *Arch. Biochem.*, 1944, **4**, 265.

† Two of the monkeys were started on one-half this intake of pteroylglutamic acid. This is equivalent to a daily dose of 55 μg and was originally reported to be adequate by Totter and associates.[10] Dr. Totter had later informed us that this intake was not adequate, so we increased it to 110 μg per day and we have found this intake to be adequate.

[10] Totter, J. R., Shukers, C. F., Kolson, J., Minus, V., and Day, P. L., *J. Biol. Chem.*, 1944, **152**, 147.

During the course of these studies careful records of the semi-weekly weights and of the daily food consumption were kept. Generally, blood was taken by venipuncture at weekly or bi-weekly intervals and used for total blood counts, for serum iron and pyridoxine analyses. At autopsy, portions of several tissues were removed for vitamin assay. The monkeys were usually placed on the diet with complete supplements for 2 to 4 weeks before withdrawal of the pyridoxine, in order to perform control tests so that each animal might serve as his own control. In addition to studies of the pyridoxine levels of the blood, periodic examinations of the metabolism of tryptophane and of carbohydrate were undertaken. Since this paper is to be confined to the studies on the blood pyridoxine levels of the monkeys, it will suffice at present to merely mention the fact that in the pyridoxine deficient monkey, as in the pyridoxine deficient dog,[7] rat,[8] and pig[9] there is a derangement of tryptophane metabolism, resulting in the excretion of xanthurenic acid.

Neurospora assay was found to lack sufficient sensitivity for our work, so we finally settled on the method of Atkins *et al.*[3] with slight modifications. The procedure for the extraction of the vitamin B_6 consisted in adding one volume of blood or plasma to 18 volumes of 0.055N H_2SO_4 (1 ml of 10N H_2SO_4 to 179 cc of H_2O) and autoclaving for 1 hour at 20 lb pressure. After cooling, the pH of the mixture was adjusted to 5.2 and the volume was made up to 25 to 30 times that of the blood used. The precipitated proteins were removed by centrifugation and the supernatant fluid was decanted. Since assay tubes containing the extracts developed turbidity following sterilization as a result of additional precipitation of protein or protein cleavage products, it was found necessary to reheat the extracts either in the autoclave for a period of 5-10 minutes at 15 lb pressure or in flowing steam for 10 minutes and to recentrifuge them. In this manner development of turbidity in the assay tubes could be avoided. The extract was now ready for testing. If the extracts were to be stored they were given a short period of sterilization and kept in the refrigerator until it was convenient to assay them. Because of the destructive effects of light, precautions were taken to keep the exposure to light at a minimum during the preparation and handling of the extracts.

As an alternate method of extraction of the vitamin we have employed takadiastase digestion in a manner similar to that described by Luckey and coworkers[11] for the extraction of pteroylglutamic acid. This has yielded, with few exceptions, substantially the same values as acid extraction. Treatment of the two sets of values by a standard statistical method showed that there was no statistically reliable difference between the data obtained with the two extraction procedures.

The growth of the test micro-organism, *Saccharomyces carlsbergensis* No. 4228, the preparation of the inoculum and the assay procedure were essentially similar to that described in the original method. A minor modification consisted in the incubation of cultures and assay tubes at room temperature (ca 25-26°) instead of at 30°. The extracts were generally assayed at levels of 2, 3 and 4 ml and growth was measured turbidimetrically with the Evelyn colorimeter using filter 620 after 16 and 18 hours of continuous agitation on a Kahn shaker. Since it was found that little was to be gained by the second set of readings, this was discontinued in many of our later analyses. Owing to the fact that extracts of blood contain some color, it was found advisable to take blank readings on the

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[7] Axebrod, H. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, 1945, **160**, 155.

[8] Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, 1943, **149**, 195.

[9] Cartwright, G. E., Wintrobe, M. M., Jones, P., Lawritsen, M., and Humphreys, S., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 35.

[11] Luckey, T. D., Briggs, G. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

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[1] Ritchey, M. G., Wicks, L. F., and Tatum, E. L., *J. Biol. Chem.*, 1947, **171**, 51.

[2] Ray, S. N., Weir, W. C., Pope, A. L., and Phillips, P. H., *J. Nutrition*, 1947, **34**, 595.

[3] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Indust. and Engr. Chem. Anal. Ed.*, 1943, **15**, 141.

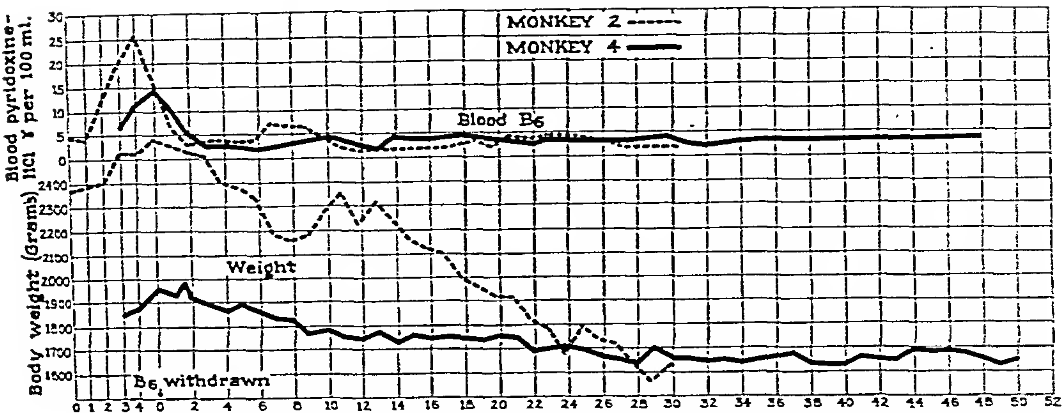
[4] Greenberg, L. D., and Rinehart, J. F., *Fed. Proc.*, 1946, **5**, 222.

[5] Rinehart, J. F., Greenberg, L. D., and Ginzton, L. L., *Blood*, 1948, in press.

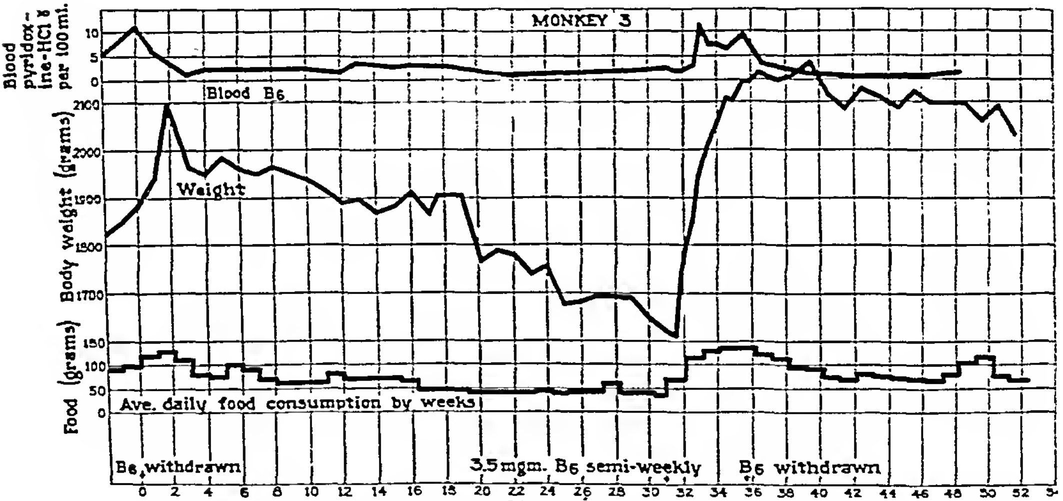
[6] Waisman, H. A., and McCall, K. B., *Arch. Biochem.*, 1944, **4**, 265.

[†] Two of the monkeys were started on one-half this intake of pteroylglutamic acid. This is equivalent to a daily dose of 55 µg and was originally reported to be adequate by Totter and associates.[10] Dr. Totter had later informed us that this intake was not adequate, so we increased it to 110 µg per day and we have found this intake to be adequate.

[10] Totter, J. R., Shukers, C. F., Kolson, J., Minus, V., and Day, P. L., *J. Biol. Chem.*, 1944, **152**, 147.



EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT AND BLOOD PYRIDOXINE
FIG. 1.



EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT, FOOD CONSUMPTION AND BLOOD PYRIDOXINE
FIG. 2.

after a period of 44 days ascorbic acid was withdrawn in connection with some other work. The curves are of the same general type as is found in our long-standing control animal (3193). It is significant that when these animals were first brought to the laboratory and placed upon the experimental diet with complete supplements, their blood pyridoxine levels were in the range of approximately 2.5 to 3.0 μg per 100 ml. During the course of 4 to 6 weeks on the diet the pyridoxine values had risen considerably above 5 μg to a range of between 8 and 18 μg. Similar observations have been made with several other monkeys.

In addition to the studies on the pyridoxine of whole blood, the distribution of the vitamin in plasma and red cells has been investigated in several instances. The results which have been obtained to date are summarized in Table I and these show that the vitamin is present in both plasma and erythrocytes. The data in the last 2 columns show that the actual blood pyridoxine concentration is in good agreement with the values calculated from the packed cell-volume, and the plasma assay tubes immediately following inoculation with the yeast.

Results. Following control observations on the experimental animals, pyridoxine was withdrawn from the diet and within 2 to 4 weeks after withholding the vitamin the monkeys ate less food and began to lose weight slowly and progressively. In most cases this weight loss continued throughout the experiment. Aside from decreased food consumption, loss in weight and some loss in vigor, the animals showed little change in outward appearance until approximately 6 to 9 months after pyridoxine deprivation when they became unkempt, sluggish in their movements and showed some signs of being hyperirritable. In addition they generally developed edema around the eyes. Similar observations have been made by McCall *et al.*[12] in the monkey. Most of the animals have shown changes in their hair and these have varied considerably from animal to animal. These alterations have consisted of thinning of the hair in some monkeys, of patches of baldness in others, of extensive loss of hair in others, while others have exhibited little change in the appearance of the coat except slight greying. The majority of the monkeys studied have usually developed fissuring and cracking of the epidermis of the hands after some 3 to 6 months of the dietary regime. A constant finding has been the development of a slowly progressive anemia. Details of this will be published later.

During the course of the experiment the pyridoxine content of the blood was followed. It was found that when monkeys were first brought into the laboratory their blood levels were usually below 5 μg per 100 cc of whole blood, but after having been on the diet with complete supplements for 2 to 3 weeks the levels increased to values ranging from 5 to 25 μg per 100 cc. Following withdrawal of the vitamin it was a matter of but a week or two until the pyridoxine of the blood had decreased to values below 5 μg, and 3 to 4 weeks following the time of withdrawal of the vitamin the levels had decreased to the neighborhood of 2 to 3 μg. As the deficiency progressed the values remained in the same range or declined still further except for a few unexplainable fluctuations. Frequently, in long-standing deficiencies we have observed values of the order of 1 μg. Two typical cases which are representative of the alterations occurring in the weight curve and in the pyridoxine content of the blood deficiency are represented graphically in Fig. I. The pyridoxine level of the blood is affected very early and it exhibits a fall before there is any appreciable loss in weight. Fig. 2 and 3 are examples of the dramatic response in the weight, food consumption and pyridoxine levels of the blood of two long-standing pyridoxine deficient monkeys following the administration of the vitamin. Monkey 3 (Fig. 2) received 3.5 mg of pyridoxine hydrochloride twice a week while monkey 1 (Fig. 3) received a supplement of 1 mg daily. The increase in weight following the administration of the vitamin was phenomenal. In addition to the functions represented in the graphs there was a return to normal of all the observed abnormalities such as blood picture, etc. Fig. 2 also shows the fall in the pyridoxine level of the blood following the withholding of the vitamin from the diet for a second time. In this instance also the fall is rapid.

For purposes of comparison the pyridoxine levels of 2 control monkeys are given in Fig. 4. Although there is considerable fluctuation in the blood pyridoxine values, it can be seen that in no case have the values fallen below 5 μg per 100 ml. In a majority of the cases the values have been above 10 μg. In the cases of some rapidly growing monkeys which we have been studying recently we found it necessary to double the pyridoxine intake in order to bring their blood pyridoxine levels above 5 μg.

The blood pyridoxine levels of 2 monkeys (A.A. deficient 3360 and 3397) which had been followed before and after deprivation of ascorbic acid are also represented in Fig. 4. The diet and supplements were the same as used for control animals with exception that

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[12] McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition*, 1946, **31**, 685.

TABLE I.
Distribution of Pyridoxine in Blood (μg pyridoxine-hydrochloride per 100 ml).

Monkey and specimen No.	Plasma pyridoxine (1)	Red cell pyridoxine (2)	Packed-cell vol. (3)	Whole blood pyridoxine observed	Whole blood pyridoxine calculated*
3327	8.9	21.1	44	13.7	14.3
6 —a	8.4	4.6	44	5.6	6.7
—b	2.5	3.9	42	3.3	3.1
8 —a	18.2	4.7	41	11.6	12.6
—b	15.3	5.7	41	9.9	11.4
—c	10.1	4.0	42	6.2	7.5
3473	5.5	5.7	47	5.4	5.6
3193 —a	10.6	4.4	42	9.4	8.0
—b	8.3	4.4	40	7.8	6.7
—c	8.0	4.0	37	6.8	6.5
—d	2.6	3.8	37	3.4	3.0
3 D†	1.9	—	—	1.9	—
4 D	2.2	1.8	41	2.3	2.0
1 D	1.2	1.8	42	1.6	1.4
11 D	2.4	3.9	44	2.9	3.1

* Calculated from columns 1, 2, and 3.
† D—Pyridoxine Deficient.

B_6 values of the animals receiving pyridoxine, except to say that it does not appear to be a question of technic, since duplicate assays yield values in close agreement. In view of the fact that Ray *et al.*[2] have shown that the blood pyridoxine levels are lowered in cobalt deficient sheep, it is possible that this fact may have some bearing on the lower values obtained in the 2 monkeys (No. 3327 and No. 3193) after a lapse of some 6 to 8 months. However, this seems rather unlikely since so far only ruminants have been shown to be susceptible to cobalt deficiency. Since the vitamin B_6 intake remained constant, the decrease in the blood pyridoxine concentration would most likely be attributable to the smaller intake of the vitamin on a weight basis resulting from the great increase in size of the animal.

Summary. Suitable modifications for the extraction and assay of blood pyridoxine are described. The vitamin B_6 levels of 14 rhesus monkeys were followed before and after withdrawal of the vitamin from the diet. The vitamin B_6 levels fell during the first 2 weeks of deprivation. This usually brought the levels down to values of 2-3 μg per 100 ml of whole blood or lower, where they remained throughout the rest of the experiment for periods exceeding 1 year. Control animals on a daily intake of equivalent to 1 mg of pyridoxine hydrochloric acid had values ranging from 5.0 to 20.8 μg, averaging 11.2. Some of the changes observed in deficient animals are discussed.

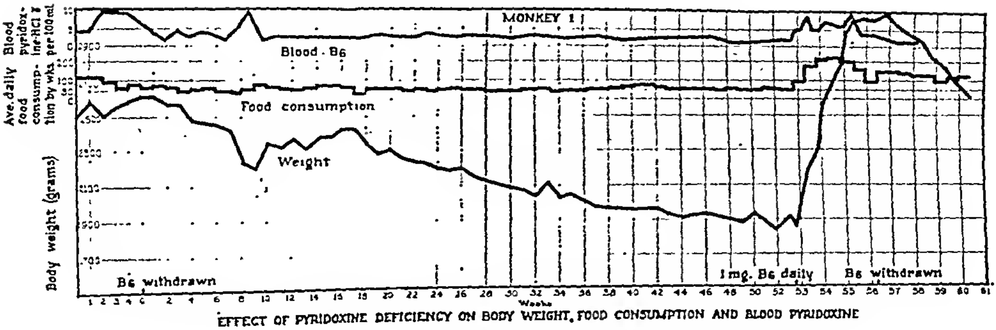

FIG. 3.

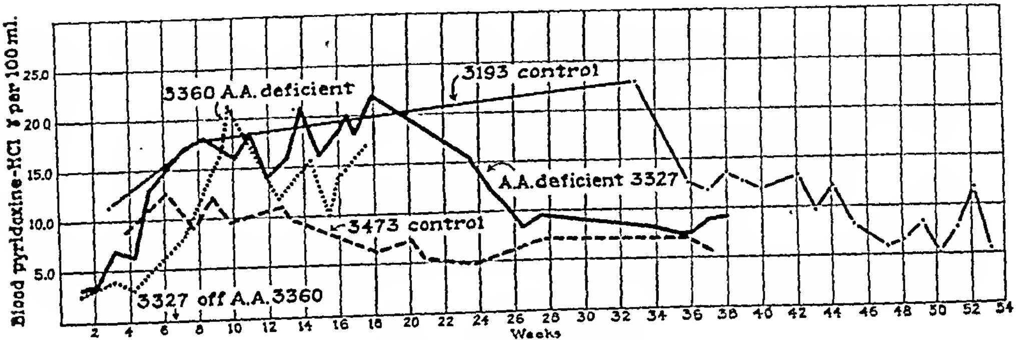

FIG. 4.

and erythrocyte concentrations of the vitamin. In monkeys on a good intake the plasma generally shows a higher concentration than the erythrocytes, but in deficient monkeys the values approach nearly equal distribution in plasma and red cells. Alterations in intake appear to influence the plasma concentration more rapidly than the erythrocyte concentration of the vitamin. The distribution of pyridoxine in the blood differs from that of thiamin, since it has been shown that approximately 90 per cent of the latter is located in the red cells. On the other hand there is a great similarity in the distribution of pyridoxine and pantothenic acid in the blood, for Pearson[13] has shown that in all the animals studied, with the exception of man, the plasma had a greater concentration of pantothenic acid than the red cells.

Discussion. The blood pyridoxine values observed are somewhat similar to those found for thiamin in the monkey.[5] The former may reach values somewhat higher than is found with thiamin, when the intakes of the 2 vitamins are of about the same order.

The average value of 42 μg per 100 ml of whole blood obtained upon mice by Ritchey *et al.*[1] is approximately twice as high as the highest values observed in our experiments. On the other hand, the results obtained by Ray and co-workers[2] on sheep are more nearly in the range of values observed by us in the monkey. They obtained an average value of 11.8 μg of pyridoxine per 100 ml of whole blood on control sheep. However, the vitamin B_6 intake of the sheep was not reported. The values on 2 control monkeys have ranged between 5.0 and 20.7 μg and have averaged 11.2 μg per 100 ml for 35 serial determinations. The average of 36 assays on the 2 monkeys used in the ascorbic acid deficiency experiment is 13.6 μg and the range is 6.1 to 21.9 μg per 100 ml of whole blood.

At present we have no adequate explanation for the wide fluctuation in the vitamin

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[13] Pearson, P. B., *J. Biol. Chem.*, 1941, 140, 423.

TABLE I.
Effect of Enterectomy on Excretion of Nicotinic Acid and Its Derivatives by Rats Following Tryptophan Administration.

	Urinary excretion, µg per 24 hr per 100 g body weight					
	Free nicotinic acid		Total nicotinic acid following acid hydrolysis		N'methyl nicotinamide	
	Avg	Range	Avg	Range	Avg	Range
Enterectomized rats						
A. Amigen	3.7	1.08- 8.1	8.1	2.24- 14.7	110	19- 240
B. Amigen + *l*-tryptophan	27	5.9 - 91	390	55 - 620	1300	250-3800
Intact rats						
C. Amigen	7.5	7.3 - 7.8	15.1	12.7 - 16.7	120	41- 270
D. Amigen + *l*-tryptophan	71	42 -100	1570	1270 -2020	1090	300-1700

rats were fasted for 24 hours before operating. The gastro-intestinal tracts from the duodenum to the anus were removed from half of the animals, and mock surgery was performed on the remainder. Morphine and light ether anaesthesia were used. After ligating and severing the superior and inferior mesenteric arteries, ligatures were placed on the small intestine, one approximately four inches from the pylorus, just below the pancreas, and another as near the rectum as possible. The gastrointestinal tract between these ligatures was removed by sectioning the gut and all attachments. Animals so treated lived from one to 4 days, when nourished by subcutaneous alimentation as described below. Secretory accumulations were removed from the stomach at 4 hour intervals with a catheter tube and syringe.

The mock surgery on the control animals resembled the enterectomy in that the viscera were handled in the same manner and then replaced intact. The medial incisions were closed with gut sutures for the muscle wall and silk thread for the skin.

The rats were then divided into 4 groups as follows: A. Enterectomized control (4 animals), B. Enterectomized plus added tryptophan (5 animals), C. Intact control (3 animals), and D. Intact animals receiving added tryptophan (3 animals). Within 1-3 hours after surgery the rats were placed in individual metabolism cages, and each received 5 ml of a sterile, modified Amigen.‡ This subcutaneous alimentation was repeated at 4 hour intervals during the entire urine collection period. The control animals in Group A and C received Amigen supplemented with 0.3% sucrose and 0.3% sodium chloride. Groups B and D received the same preparation containing 10 mg of L-tryptophan per ml. During the 24 hour period a total of 6 injections were given for a total dosage of 30 ml for each rat. Autopsy following the experiment showed that the solution was completely absorbed from beneath the skin of the back and neck of each animal.

At the end of the 24 hour collection period the urines were made to volume, filtered, and stored at 5°C under toluene until analyzed. N'Methyl nicotinamide was determined by the acetone-fluorometric method.[9] While this method gave reproducible results in previous studies with urine from normal rats, the results were more variable with these urines, particularly when large quantities of tryptophan were given.

Another portion of each urine sample was neutralized, diluted and analyzed for nicotinic acid by the microbiological assay.[11] The value obtained was termed "free" niacin. A third portion of the urine was hydrolyzed by autoclaving for one hour at 15 lb pressure with an equal volume of 2 N HCl. After neutralizing, "total" niacin was determined microbiologically.[11] The identity and signifi-

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‡ Mead Johnson preparation containing 5% enzymatic digest of casein and 5% glucose.

[11] Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem., Anal. Ed.*, 1943, 15, 471.

16812

Effect of Enterectomy on Synthesis of Niacin in the Rat.*

L. M. Henderson† and L. V. Hankes. (Introduced by C. A. Elvehjem.)

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

The limited interchangeability of tryptophan and niacin in the nutrition of experimental animals[1] receiving low protein rations has indicated the formation of niacin from this amino acid.[2-4] This synthesis through the intermediate, kynurenine, has been shown to occur in *Neurospora*.[5] Investigations of Ellinger *et al.*,[6] and the marked effect of the character of the carbohydrate on the growth of rats receiving a niacin-low diet containing 9% casein[1] suggested a possible transformation of tryptophan to niacin or its derivatives by intestinal bacteria. The chief arguments against this site of synthesis were the prompt excretion of niacin derivatives in the urine following parenteral administration of tryptophan[7] and more recently the evidence that injection of free tryptophan into the egg results in increased niacin in the chick embryo.[8] A study of the effect of removal of the major portion of the gastro-intestinal tract on the capacity of the animal to form niacin derivatives and excrete them in the urine appeared to be a direct and plausible approach to this problem.

Surgical procedure and ease of catheterization for collection of urine specimens for short periods made the dog preferable to the rat. It was found, however, that the responses to tryptophan in the dog were small compared to those in the rat. N'Methyl nicotinamide was elevated 2-4 fold following the administration of a large dose of tryptophan to the dog, but the acetone-fluorometric method[9] was not suitable in our hands for accurate determination of this metabolite. The amount present was small necessitating the use of large samples, which resulted in high blanks and poor recoveries. Small increases were noted in the nicotinic acid values for dog urine following trytophan administration. The largest increase was noted in the niacin released by acid hydrolysis,[3] but the increase was never more than 100%. Increases up to 100 fold were found in this fraction in rat urine following administration of L-tryptophan either orally or parenterally. The dog appears to have a limited capacity for "converting" tryptophan to niacin derivatives, which is in agreement with the finding that black tongue can be produced with rations containing 19% casein.[10]

Experimental. Male, Sprague-Dawley rats weighing 235-360 g were employed. After receiving stock ration for several weeks, the

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† Present address: Department of Chemistry, University of Illinois, Urbana, Ill.

[1] Krehl, W. A., Sarma, P. S., Teply, L. J., and Elvehjem, C. A., *J. Nutrition*, 1946, **31**, 85.

[2] Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1946, **163**, 343.

[3] Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573.

[4] Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

[5] Beadle, G. W., Mitchell, H. K., and Nye, J. F., *Nat. Acad. Sci.*, 1947, **33**, 155.

[6] Ellinger, P., Abdel Kader, M. M., and Emmanuelowa, A., *Brit. J. Exp. Path.*, 1947, **28**, 261.

[7] Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

[8] Schweigert, B. S., German, H. L., and Garber, M. J., *J. Biol. Chem.*, 1948, **174**, 383.

[9] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, **167**, 157.

[10] Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **158**, 173.

TABLE I.
Effect of Enterectomy on Excretion of Nicotinic Acid and Its Derivatives by Rats Following Tryptophan Administration.

	Urinary excretion, μg per 24 hr per 100 g body weight					
	Free nicotinic acid		Total nicotinic acid following acid hydrolysis		N'methyl nicotinamide	
	Avg	Range	Avg	Range	Avg	Range
Enterectomized rats						
A. Amigen	3.7	1.08- 8.1	8.1	2.24- 14.7	110	19- 240
B. Amigen + *l*-tryptophan	27	5.9 - 91	390	55 - 620	1300	250-3800
Intact rats						
C. Amigen	7.5	7.3 - 7.8	15.1	12.7 - 16.7	120	41- 270
D. Amigen + *l*-tryptophan	71	42 -100	1570	1270 -2020	1090	300-1700

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16812

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L. M. HENDERSON[†] AND L. V. HANKES. (Introduced by C. A. Elvehjem.)

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[4] Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

[5] Beadle, G. W., Mitchell, H. K., and Nye, J. F., *Nat. Acad. Sci.*, 1947, **33**, 155.

[6] Ellinger, P., Abdel Kader, M. M., and Emmanuelowa, A., *Brit. J. Exp. Path.*, 1947, **28**, 261.

[7] Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

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[9] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, **167**, 157.

[10] Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **158**, 173.

16813

Mucolytic Enzyme Systems. V. Anaphylactic Effects on Hyaluronidase Inhibitor in Serum of Normal and Herpetic Rabbits.*

DAVID GLICK AND BERRY CAMPBELL.†

From the Departments of Physiological Chemistry and Anatomy, University of Minnesota Medical School, Minneapolis.

In an earlier study,[1] it was demonstrated that poliomyelitis infections provoked a significant elevation in the concentration of hyaluronidase inhibitor in blood serum. Good and Campbell[2] showed that anaphylactic shock can precipitate an active encephalitis in rabbits in which *Herpes simplex* virus infection is latent after recovery from an attack of the disease. Accordingly, an investigation was undertaken to determine (1) the effect of *Herpes* infection on the level of the hyaluronidase inhibitor in the blood serum of rabbits,

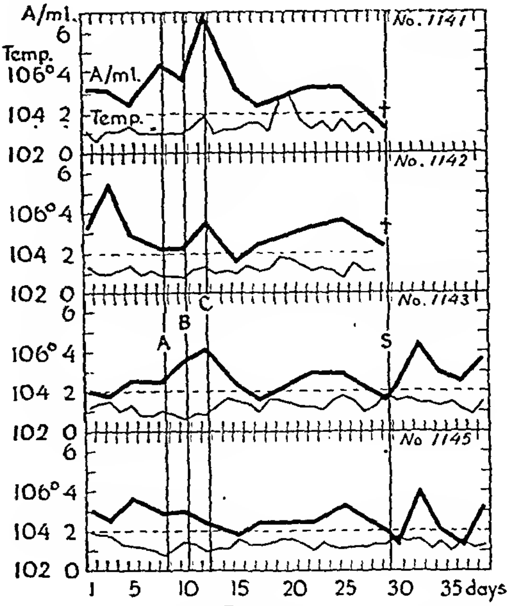

FIG. 1.

Effect of sensitization to egg white, and shock, on the level of hyaluronidase inhibitor in rabbit serum. A, B, C represent the time of sensitizing injections and S the time of the shock injection.

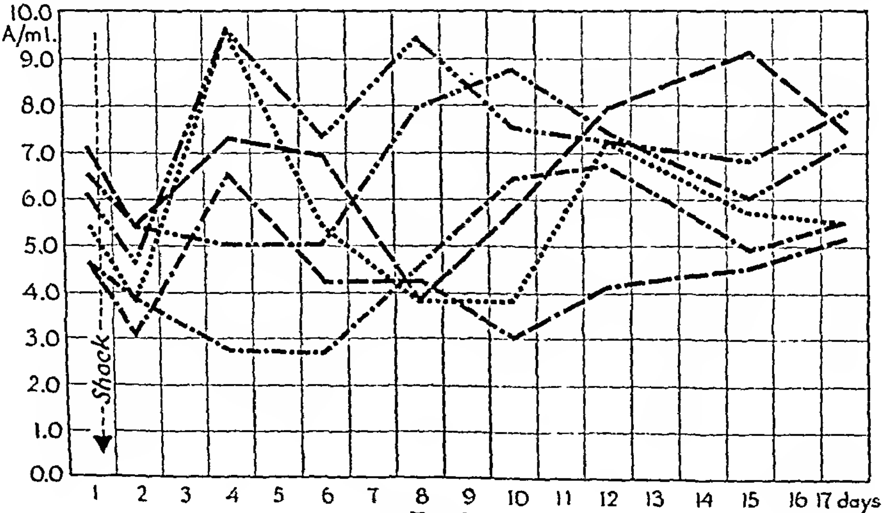

FIG. 2.

Effect of anaphylactic shock on the level of hyaluronidase inhibitor in rabbit serum.

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† The authors are grateful for the assistance rendered by Dr. R. A. Good, and the technical aid afforded by Mr. P. Edmondson and Mrs. W. Olsen.

[1] Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, **83**, 200.

[2] Good, R. A., and Campbell, B., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 82.

cance of the substance(s) which gives rise to niacin activity for *L. arabinosus* during acid hydrolysis is unknown.[3] The activity of a number of niacin related compounds for *L. arabinosus* have been reviewed by Snell.[12]

Results and discussion. The N′methyl nicotinamide content of the urines (Table I), though variable, was approximately 10 times as high for the rats receiving tryptophan. Enterectomy had no significant effect on this response to tryptophan administration. The average daily excretion of "free" niacin was also increased following tryptophan administration from 3.7 to 27 µg per 100 g of body weight for the enterectomized rats and from 7.5 to 71 µg for the intact animals. Except for one animal whose "free" niacin excretion was 91 µg all of the rats in Group B excreted less than 17 µg, while the intact animals in Group D excreted 42 to 100 µg.

The "total" niacin values increased 100 fold in the intact animals and approximately 50 fold in the enterectomized rats. It should be pointed out that the Amigen administered contains a small amount of tryptophan. The added L-tryptophan, however, resulted in a 15 to 20 fold increase in the total tryptophan intake. At the low tryptophan level (Groups A and C) the N′methyl nicotinamide excretion was much greater than total nicotinic acid. The effect of massive doses of tryptophan was most marked in the latter fraction, resulting in a greater excretion of "total" niacin than N′methyl nicotinamide in Group D. Less than 1% of the administered tryptophan appeared in the urine as the end products measured.

It is evident that the increase in "free" and "total" niacin in the urine following tryptophan administration is somewhat less marked in the enterectomized animals than in intact controls. Whether this difference is a result of the action of bacteria, possibly on tryptophan which has passed into the gut, or of removal of intestinal tissue which participates in the formation of these metabolites, or of the effect of surgical trauma on the metabolic activity of other tissues is not known. It is evident, however, that significant though slightly limited formation of nicotinic acid end products occurred in the enterectomized animals. It seems unlikely that the microbial population in the stomach and a few inches of the intestine could contribute much to urinary nicotinic acid derivatives. Some growth did occur, however, since the stomach contents had a pH of 8-8.5, and there was evidence of putrefactive changes after 2-3 days. It is interesting that enterectomy did not affect the N′methyl nicotinamide excretion, since it has been found to be synthesized in the liver of the rat.[13] The data presented suggest that the tissues, and not the intestinal microflora are the primary sites of formation of these compounds in response to the administration of large amounts of tryptophan. The possibility of the formation of small, though physiologically significant, amounts of niacin derivatives in the tract has not been eliminated.

Summary. 1. The effect of removal of the intestinal tract below the pancreas on the ability of the rat to form niacin derivatives in response to tryptophan administered subcutaneously has been determined.

2. The response in urinary N′methyl nicotinamide was not affected by enterectomy.

3. "Free" and "total" urinary niacin as measured by microbiological assay before and after acid hydrolysis respectively were increased in enterectomized animals although the increase was not as great as in intact animals.

4. The results suggest that the tissues are the primary site of the formation of niacin derivatives in the rat in response to large doses of tryptophan.

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[12] Snell, E. E., *Biological Symposia*, 1947, **12**, 183.

[13] Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, **150**, 401.

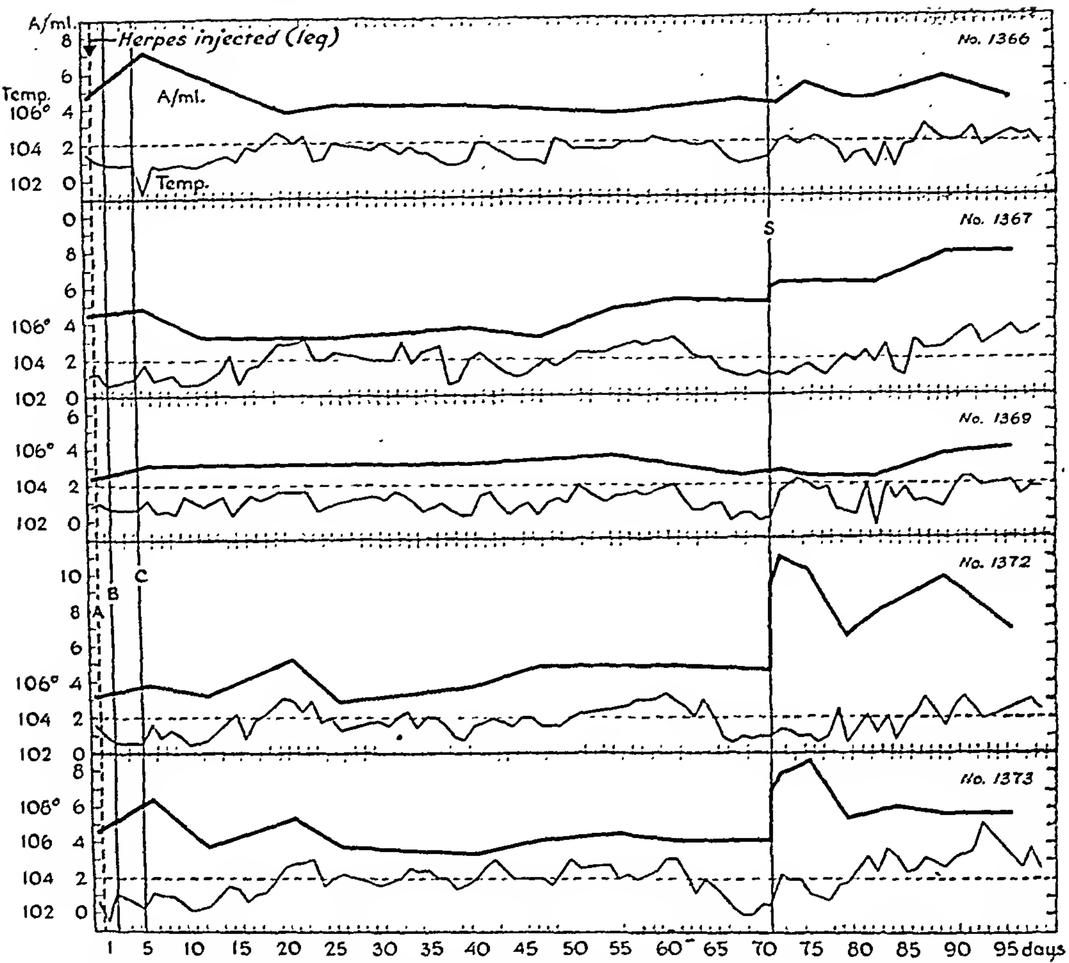


FIG. 4.

Effect of herpetic infection via the leg and subsequent anaphylactic shock on the level of hyaluronidase inhibitor in rabbit serum. A, B, C represent the time of sensitizing injections and S the time of the shock injection.

In the procedure employed, 0.05 cc of serum was required for each determination. The anaphylaxis was effected by sensitizing the rabbits to injections as follows: 1 cc i.v., first day; 0.5 cc i.v., third day; 1 cc i.m., fifth day; and shocking with 0.6 cc i.v. Infections were induced through both the corneal and leg routes. The former were effected by flooding the scarified cornea with a 10% suspension of herpetic mouse brain in physiological saline solution, and the latter by injection of 0.5 cc of the brain suspension into the quadriceps muscle of the right leg.

Results and discussion. The effect of anaphylaxis *per se* on the hyaluronidase inhibitor in blood serum is shown in Fig. 1. The variations which occurred before and during the period of sensitization to the egg white followed no consistent pattern and were of small magnitude. Shock killed 2 of the animals, and in the two that survived, a small elevation in the inhibitor concentration was produced which cannot be considered significant. No consistent effect was observed as a consequence of shocking 6 additional animals. Fig. 2. A small depression was observed the second day after shock, but the magnitude of this effect was slight, and the subsequent changes were widely variable.

Infection through the corneal route pro-

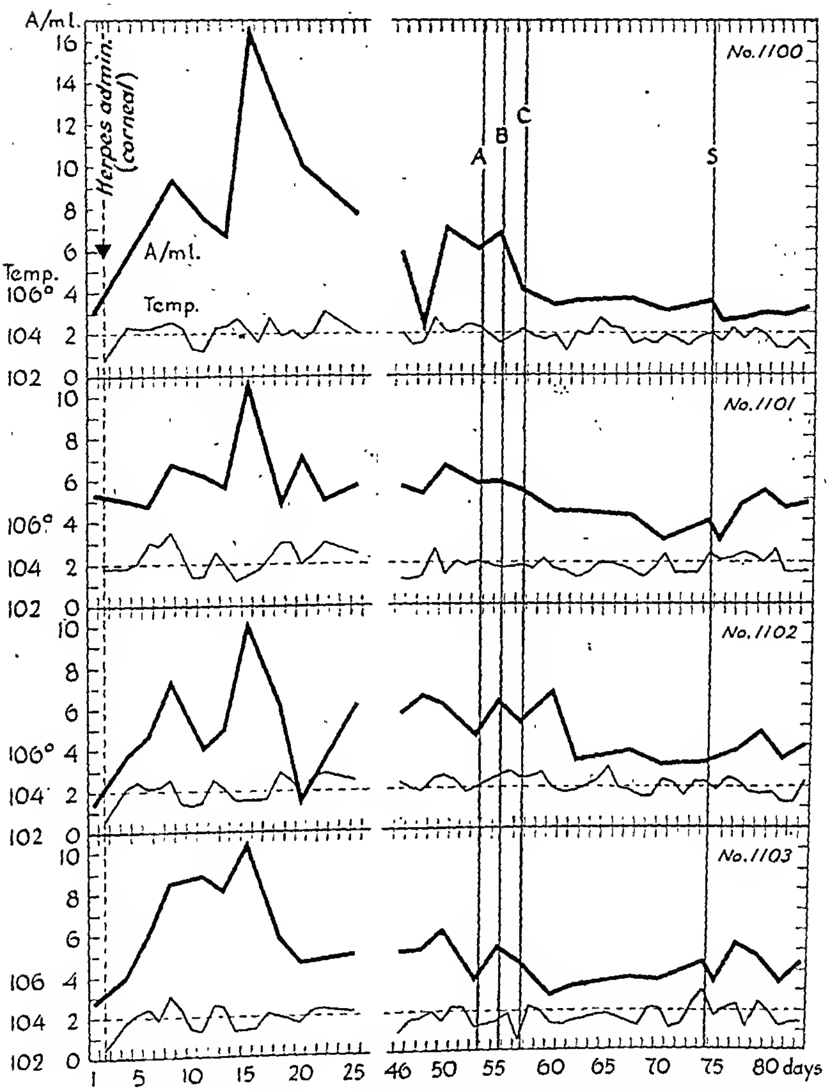
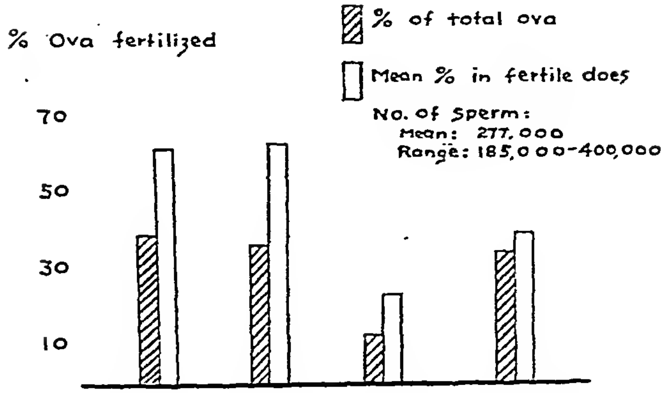


FIG. 3.

Effect of herpetic infection via the cornea, and subsequent sensitization to egg white and shock on the level of hyaluronidase inhibitor in rabbit serum. A, B, C represent the time of sensitizing injections and S the time of the shock injection.

(2) whether a precipitation of active encephalitis by anaphylaxis will be accompanied by a change in the level of the inhibitor, and (3) if an alteration in the latter will result from anaphylaxis *per se*.

Experimental. The preparation of hyaluronic acid from human umbilical cords and hyaluronidase from bull testes, and the viscosimetric measurement of hyaluronidase inhibition by serum were carried out as previously described.[1] The term employed to express the inhibiting property of the serum is $\frac{R - R_0}{R_0}$, designated as (A), where (R_0) represents the time in seconds for the viscosity of the reaction mixture to fall to one-half its initial value, and (R) is the corresponding term for the fall in viscosity in the presence of serum.



Treatment	Human Sem. plasma	Rabbit Sem. plasma	Bull Sem. plasma	Fructose Ringer Solution
No. of does	16	14	14	16
Sterile does	7	4	7	4
Total ova	498	412	517	507

FIG. 1.

Effects of heterologous seminal plasma on fertilizing capacity of rabbit spermatozoa (immediate insemination).

intervals of 7 days, was used throughout this investigation. About 0.01 to 0.03 ml of semen, depending on the concentration of sperm, was first suspended in 10 ml of Fructose Ringer solution (NaCl, 0.85 mg; KCl, 0.042 g; $CaCl_2$, 0.024 mg; $MgCl_2$, 0.02 g; $NaHCO_3$, 0.1 g; Fructose, 0.1 g; Double glass distilled water, 100 ml). The sperm concentration of this suspension was immediately and quickly examined by means of a haemocytometer technic in order to obtain a minimal effective number of sperm (Number of spermatozoa required to fertilize only a portion of the ova ovulated). Then 0.5 ml of this suspension was added to 0.5 ml of human, bull, rabbit seminal plasma, or to 0.5 ml of Fructose Ringer solution which was used as a control. The mixture (all 1 ml in volume) was inseminated, immediately in the first series of experiments, or after storage at room temperature for one hour in the second series of experiments. After insemination, the number of spermatozoa was counted again 4 to 6 times to obtain an accurate number of spermatozoa inseminated.

Six to 8 doe rabbits (superovulated according to Pincus[4]) were used at a time. Two does each were inseminated with the same sperm mixture. The ovulation injection of pituitary extract was given just after insemination. The animals were sacrificed about 25 hours later. The ova of each rabbit were flushed from fallopian tubes with undiluted rabbit serum and the fertilized and unfertilized ova were counted.

In a third series of experiments, sperm cells of human, bull or rabbit were obtained by centrifugation of semen samples and then resuspended in saline and centrifuged again. The washed sperm cells were deep frozen with a mixture containing solid carbon dioxide and acetone and then suspended in Fructose Ringer solution (about 800 millions of dead sperm per ml). A minimal effective number of rabbit spermatozoa was suspended in this fluid and stored at room temperature for one hour before insemination. In this series, one part of fresh egg yolk was suspended into one part of Fructose Ringer solution as a control.

Results: Fig. 1-3 illustrate the results. The fertilizing capacity of spermatozoa under different treatments was expressed in terms of percentage of ova fertilized. A few of the does (31%) had no ova fertilized, due to female infertility and/or to the small number duces an acute systemic reaction in the rabbit which can be followed by means of the animal's temperature. Temperatures exceeding 104° are considered definitely pathognomonic. In animals which survive the disease it is usually difficult to precipitate it again, or as severely, by shock, compared to those which survive after infection through the leg route. On the other hand, the latter group do not usually develop the acute symptoms and high fever that characterize the former during the initial sickness.

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[4] Pincus, G., *Anat. Rec.*, 1940, 77, 1.

These influences are reflected in the changes in the inhibitor concentration. Thus, in Fig. 3, the effect of the corneal infection can be seen to produce a consistent elevation which has a characteristic double peak during the period of acute illness. After recovery, sensitization and shock failed to cause important changes in either the infective state or the inhibitor level.

When the virus was injected into the leg, no large increases in the inhibitor concentration were found, Fig. 4, and the animals were not acutely ill. The sensitization was begun on the same day that the rabbits were infected in order to save time, as these experiments continue for months. From Figs. 1 and 3, it is apparent that sensitization *per se* has no appreciable influence on the inhibitor level. A prompt elevation of the inhibitor level followed shock in those animals in which precipitation of the disease occurred. Animals No. 1372, 1373 showed the most severe symptoms, No. 1366 little effect, and No. 1369 none. The changes in the inhibitor show a correlation with these symptomatic effects.

Summary. Hyaluronidase inhibitor in the blood serum of the rabbit was found to undergo no consistent change in concentration as the result of anaphylaxis induced by egg white. An elevation of the inhibitor level was observed during the acute phase of infection by *Herpes simplex* virus administered via the corneal route. Anaphylactic shock in rabbits which had recovered from the herpetic infection, administered via the leg route, resulted in elevated inhibitor values which were correlated with the severity of the disease thus precipitated from the latent state by the shock.

16814

Effects of Heterologous Seminal Plasma and Sperm Cells on Fertilizing Capacity of Rabbit Spermatozoa.

M. C. CHANG. (Introduced by D. Rapport.)

From the Worcester Foundation for Experimental Biology, Shrewsbury, Mass., and the Department of Physiology, Tufts Medical School, Boston, Mass.

In a previous experiment,[1] the beneficial effects of rabbit seminal plasma on the fertilizing capacity of rabbit spermatozoa was demonstrated. This paper reports the effects of human, bull, and rabbit seminal plasma, as well as of dead sperm of these species, on the fertilizing capacity of rabbit spermatozoa.

Methods: Human semen was collected into a sterile tube and stored at 2°C no more than 12 hours. Bull semen and vasectomized rabbit semen were obtained by means of an artificial vagina[2,3] about 1 to 2 hours before use. Human and bull samples were centrifuged at 2,000 R.P.M. for about 30 to 40 minutes and the supernatant fluid was used as seminal plasma.

The semen of a single rabbit, collected at tion of the mixture. The percentage of ova fertilized was low when rabbit sperm were suspended in bull seminal plasma or in Fructose Ringer solution. Statistical analysis of the data (by t test) however, shows that there is no significant difference between human and rabbit seminal plasma and Fructose Ringer solution, but there is a significant difference between bull seminal plasma and other sperm mixtures ($t = 2.3$, 2.78, or 2.4, $p < .05 > .01$). This clearly demonstrates that bull seminal plasma has an adverse effect on the fertilizing capacity of rabbit sperm.

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[1] Chang, M. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 51.

[2] Walton, A., Notes on Artificial Insemination of Sheep, Cattle and Horses. 1942, London: Holborn Instrument Co.

[3] Macirone, C., and Walton, A., *J. Agri. Sci.*, 1938, **28**, 122.

Fig. 2 illustrates the results of another 32 inseminations performed when the sperm mixtures were allowed to stand for one hour before insemination. The beneficial effects of human or rabbit seminal plasma as compared with Fructose Ringer solution or bull seminal plasma is clearly shown. There is a very significant difference between human and bull seminal plasma ($t = 3.9$, $p < .01$), human and Ringer solution ($t = 3.55$, $p < .01$), rabbit and bull plasma ($t = 3.75$, $p < .01$) and rabbit and Ringer solution ($t = 3.12$, $p < .01$).

It is clear by comparison of Fig. 1 and 2, that human and rabbit seminal plasma have a beneficial effect, while bull seminal plasma has an immediate harmful effect, on the fertilizing capacity of rabbit spermatozoa. Fructose Ringer solution has no immediate ill effect, but the fertilizing capacity of rabbit spermatozoa decreases after one hour in Fructose Ringer solution.

Fig. 3 illustrates the result of another 34 inseminations when the minimal effective number of rabbit spermatozoa were suspended in Fructose Ringer solution containing dead human, bull and rabbit sperm cells or egg yolk. The percentage of ova fertilized was high when rabbit spermatozoa were mixed with human or bull sperm cells, but it was rather low when mixed with rabbit sperm cells or with egg yolk. There is a significant difference between human sperm cells and egg yolk ($t = 2.8$, $p < .02 > .01$); the difference between other treatments is not significant. Since egg yolk is considered the best medium for the preservation of fertilizing capacity of spermatozoa in the practice of artificial insemination,[5] it is of interest to note that sperm cells of human and bull are better than or equal to egg yolk.

The results presented in Fig. 2 and 3 are comparable because the sperm mixtures were stored for one hour before insemination. In this respect, human, rabbit, or bull sperm cells and human or rabbit seminal plasma are beneficial to the fertilizing capacity of rabbit spermatozoa (No statistical significant difference) while bull sperm cells are much better than bull seminal plasma (55% with significant difference). Fructose Ringer solution with egg yolk is definitely better for the preservation of fertilizing capacity than without egg yolk (28%, with significant difference). Human sperm cells are better than Fructose Ringer solution containing egg yolk (39%, with significant difference).

Discussion. In the previous experiment,[1] the beneficial effect of seminal plasma was demonstrated when 0.9% NaCl was used as control. It was thought that 0.9% of NaCl may have an adverse effect on spermatozoa. The present study once again demonstrated the same fact when a balanced salt solution with the metabolic substrate, fructose[6] was used.

In a study of the effects of high dilution on fertilizing capacity[7] Chang has postulated that there might be a beneficial substance in the seminal plasma or in the spermatozoa. The present study clearly verifies this assumption. Whether the beneficial effect of seminal plasma or egg yolk is to prevent the escape of essential substances in the spermatozoa as suggested by Emmens and Swyer[8] or to contribute an essential substance for the prolongation of fertilizing capacity is hard to say at present. If sperm cells, seminal plasma or egg yolk contribute this essential substance, the sources of this substance may be widely distributed in animal tissues. Consid-

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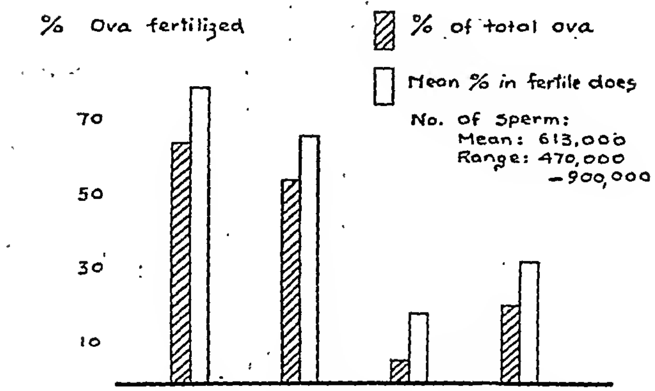
[5] Anderson, J., The Semen of Animals and Its Use in Artificial Insemination. 1947: Edinburgh, Genetic Institute.

[6] Mann, T., *Nature*, 1946, **157**, 79.

[7] Chang, M. C., *Science*, 1946, **104**, 361.

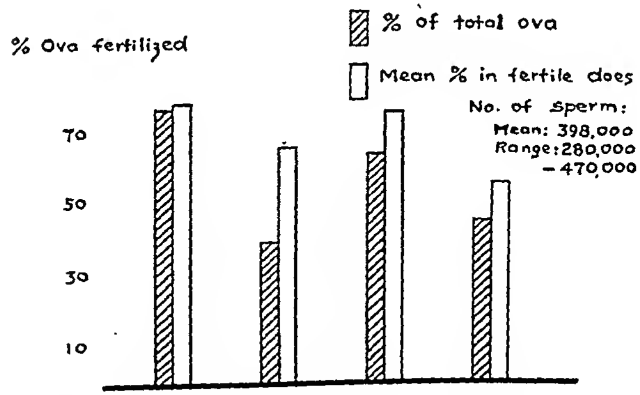
[8] Emmens, C. W., and Swyer, G. I. M., *Nature*, 1947, **160**, 718.



Treatment	Human Sem. plasma	Rabbit Sem. plasma	Bull Sem plasma	Fructose Ringer Solution
No of does	8	8	8	8
Sterile does	1	1	3	5
Total ova	179	191	372	239

FIG. 2.

Effects of heterologous seminal plasma on fertilizing capacity of rabbit spermatozoa (stored one hour before insemination).



Treatment	Human Sperm	Rabbit Sperm	Bull Sperm	Egg yolk
No. of does	6	10	8	10
Sterile does	0	3	1	3
Total ova	145	273	208	303

FIG. 3.

Effects of dead sperm of different species on fertilizing capacity of rabbit spermatozoa (stored for one hour before insemination).

of sperm inseminated. Thus, the percentages of all ova, including the ova of sterile does, in each group and the mean percentage of ova fertilized in the fertile does were presented in figures. In the calculation of statistical significance between each group, the mean percentages of fertilized ova of all does, including sterile does, was taken.

Fig. 1 illustrates the results of 60 inseminations performed immediately after prepara-

TABLE I.
Occurrence of Streptomycn-Resistant Tubercle Bacilli in Mice Treated with Streptomycin.

No. of mice	Amt streptomycin sulphate in mg per day	% mortality	Avg survival time in days*	No. of mice which developed streptomycin resistant strains			Range of sensitivity to streptomycin sulphate† (in mg per ml of medium)
				Total %	Sacrificed %	Died %	
19	1500	26.3	131.6	17 (89.4)	14 (100)	3 (60)	12.5 to >1000
20	750	85	73.5	13 (65.0)	3 (100)	10 (58.8)	12.5 to >1000
18	375	100	40.1	4 (22.2)	0	4 (22.2)	12.5 to 500
18	187.5	100	19.7	1 (5.5)	0	1 (5.5)	12.5
20	0	100	14.6	0	0	0	0.19 to 1.56

* Includes mice sacrificed at 155 days.

† Indicates difference in sensitivity to streptomycin of cultures from individual mice. No significant difference in range of sensitivity was noted between mice sacrificed and those that died.

shown readily became resistant *in vitro*.[3] This culture, however, was sensitive *in vitro* to between 0.19 and 1.56 µg per ml of streptomycin sulphate.[3] The mice were divided into groups and treated by subcutaneous injection twice daily with one-half the amounts of streptomycin shown in Table I. Experimental attrition reduced the number of mice on which complete data were obtained to 95. Treatment was continued for a period of 155 days, at the end of which time, surviving mice were sacrificed. At the time each mouse died, or was sacrificed, it was autopsied, and one lung fixed in 3.7% formaldehyde, and gross and microscopic examination made as previously described.[8-11] Isolation of tubercle bacilli was accomplished by grinding the other lung in a sterile mortar, and treating the residue with oxalic acid, as described by Corper and Uyei,[12] and planting on Herrold's medium.[13] When sufficient growth was obtained, these cultures were tested in serum synthetic liquid media for their sensitivity to streptomycin.

Results. Table I shows the results obtained. It is readily apparent that a large proportion of the mice developed streptomycin resistant tubercle bacilli. The incidence of the occurrence of streptomycin resistant strains of tubercle bacilli in the different groups of mice was directly related to the amount of streptomycin administered. A similar relationship existed between the development of resistance and the average survival time. The two smaller doses of streptomycin apparently were not sufficiently large to suppress the infection for a period which would permit the multiplication of the resistant organisms to a detectable number.

Results essentially similar to these have also been obtained with H37Rv strain of *M. tuberculosis*.

In many of the mice, the pathological findings confirmed the bacteriological evidence of the presence of streptomycin resistant tubercle bacilli. Evidence of active necrotic disease superimposed upon healed or healing areas was common in many of the animals from which streptomycin resistant tubercle bacilli were isolated.

Since we have found that streptomycin resistant tubercle bacilli appear in a large proportion of mice treated with adequate amounts of streptomycin, an experimental method is now available for the determination of the effect of various factors, including combined therapy, on the development of resistance to streptomycin *in vivo*.

Summary. Streptomycin resistant tubercle bacilli were obtained from the majority of intravenously tubercularized mice treated with amounts of streptomycin which permitted a relatively long survival time.

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[8] Youmans, G. P., and McCarter, J. C., *Am. Rev. Tuberc.*, 1946, **52**, 432.

[9] Raleigh, G. W., and Youmans, G. P., *J. Inf. Dis.*, 1948, **82**, 197.

[10] Raleigh, G. W., and Youmans, G. P., *J. Inf. Dis.*, 1948, **82**, 205.

[11] Youmans, G. P., and Raleigh, G. W., *J. Inf. Dis.*, 1948, **82**, 221.

[12] Corper, H. J., and Uyei, N., *J. Lab. and Clin. Med.*, 1930, **15**, 348.

[13] Herrold, R. D., *J. Inf. Dis.*, 1931, **48**, 236.

ering the beneficial effect of bull sperm cells on rabbit spermatozoa and the harmful effect of bull seminal plasma, the presence of this essential substance in the spermatozoa of different species rather than in the seminal plasma is indicated.

It is of biological interest to note the compatibility of rabbit sperm and human seminal plasma and the incompatibility of rabbit sperm and bull seminal plasma.

A high dilution of sperm was employed in the present study in order to obtain a minimal effective number of spermatozoa. It was observed that the progressive movement of sperm lasted for 5 to 6 hours in seminal plasma (only 3 to 4 hours in bull seminal plasma) or in the solutions containing added dead sperm. But only sluggish movement was observed in Fructose Ringer solution from the beginning for 2 to 3 hours.

Summary. Superovulated does were inseminated with a minimal effective number of sperm suspended in Fructose Ringer, or seminal plasma of human, bull or rabbit; or in Fructose Ringer suspension of egg yolk, or deep frozen sperm. It was found that the seminal plasma of human or rabbit had a beneficial effect while that of bull had an ill effect on the fertilizing capacity of rabbit sperm as compared with Fructose Ringer. Fructose Ringer containing dead sperm of human, rabbit or bull was found as good or better media for the preservation of sperm as egg yolk; which indicates the presence of essential substances in the sperm cells for the maintenance of their fertilizing capacity.

The writer wishes to acknowledge his gratitude to Dr. G. Pincus for encouragement, to Mr. C. Putney of Massachusetts Selective Breeding Association for supplying bull semen, to Mr. R. Gunnarson for assistance, to the Foundation for Applied Research, San Antonio, Texas, and the Committee of Human Reproduction of National Research Council for Grant in Aid.

16815 P

Occurrence of Streptomycin Resistant Tubercle Bacilli in Mice Treated with Streptomycin.*

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Tubercle bacilli readily become resistant to streptomycin *in vitro*[1-3] and in patients being treated with streptomycin.[1,4-6] Feldman, Karlson and Hinshaw[7] reported the isolation of streptomycin resistant tubercle bacilli from 3 of 8 guinea pigs treated for a prolonged period with 6.0 mg streptomycin per day, after these had been infected with a streptomycin sensitive strain of *M. tuberculosis*.

Methods. One hundred mice were infected intravenously with 1.0 mg of a suspension of a culture of *M. tuberculosis* var. hominis (strain no. 24)[3] which we had previously

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[*] This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meetings, Mayo Clinic*, 1946, **21**, 126.

[2] Middlebrook, G., and Yegian, D., *Am. Rev. Tuberc.*, 1946, **54**, 553.

[3] Williston, E. H., and Youmans, G. P., *Am. Rev. Tuberc.*, 1947, **55**, 536.

[4] Youmans, G. P., and Karlson, A. G., *Am. Rev. Tuberc.*, 1947, **55**, 529.

[5] Streptomycin Committee, Veterans Administration, Report to the Council on Phar. and Chem., *J.A.M.A.*, 1947, **135**, 634.

[6] Streptomycin Committee, Veterans Administration, Report to the Council on Phar. and Chem., *J.A.M.A.*, 1948, **138**, 584.

[7] Feldman, W. H., Karlson, A. G., and Hinshaw, H. C., *Am. Rev. Tuberc.*, 1948, **57**, 162.

Cu. The tissues were at a distance of 50 cm from the target. The studies reported here in detail involved doses of 500 r or less at a dose rate of about 25 r per minute. Some studies were also made using doses up to 10,000 r of unfiltered radiation at a higher dose rate.

Results. Explants given small doses of X-rays had a shorter latent period than unirradiated controls. This seems to be true for doses of X-rays at least as high as 500 r.

Data for two representative experiments are shown in the graphs, Fig. 1, which indicate the percentage of the explants which show one or more migrating cells as a function of the time after irradiation for various doses of X-rays. Typical sigmoid curves are obtained.

In order to compare the effects of different doses of radiation more easily, we have plotted in Fig. 2 data obtained from graphs like those in Fig. 1, giving the time after irradiation at which 50% of the explants show one or more migrating cells as a function of the dose of X-rays for the above experiments. Different heart preparations have different latent periods for both controls and irradiated explants. A fact which is consistent among all preparations, however, is that the minimum latent period occurs at a dose of about 30 r. At higher doses of irradiation the latent period increases slowly with dose and it is not until explants are irradiated with 1,000 r that the latent period is as long as in the controls. With doses of 10,000 r the latent period is approximately 50% longer than in the controls, but the result may not be strictly comparable since the dose rate was used for the 10,000 r is considerably higher than that with smaller doses.

One experiment using 11-day old embryonic chick heart fragments gave results similar to those obtained with adult heart tissue, although as is characteristic of embryonic tissue the latent periods were very short. The latent period was less than 2 hours for 30 r, approximately 3 to 4 hours for controls and 5 hours for 5,000 r.

Our data in Fig. 2 indicate that irradiation has two apparent effects on the duration of the latent period. At doses below 30 r the most obvious effect is a stimulating effect on the migration of cells from a fresh explant. With doses of X-rays higher than 30 r the gradually increasing latent period suggests a superimposed inhibition which increases with the dose of radiation. This inhibition of migration with larger doses of X-ray is not surprising since practically all the older literature shows that irradiation of cell cultures causes an inhibition which becomes evident only after a delay of approximately 24 hours. The stimulating effect of small doses of irradiation is somewhat more surprising. Ludford[4] observed in a few cases a stimulating effect of radiation on actively growing cultures but made no systematic and controlled study. He also observed that in growing cultures macrophages seem to inhibit the growth of fibroblasts. In these cultures the growth stimulating effect of irradiation was attributed to the inhibition of the macrophage activity by X-rays. In our experiments on fresh explants, however, the mechanism responsible for the stimulation or inhibition may be different.

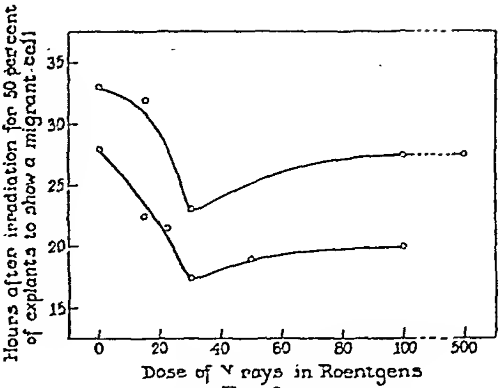

FIG. 2.

Curves showing how the time for 50% of explants to show one or more migrating cells depends on the dose of X-rays expressed in roentgens.

Another explanation which has been offered[5] is that radiation damages some cells, causing a release of nutrients which can stimulate other cells into activity. This occurs with considerably larger doses of radiation than those used by us.

Our results may be of interest in connec-

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[4] Ludford, R. S., *Proc. Roy. Soc.*, 1934, **115**, 278.

[5] Giese, A. C., *Quart. Rev. Biol.*, 1947, **22**, 253.

16816

Effect of X-rays on the Migration of Cells from Adult Tissue Explants.*

R. S. Hoffman and S. H. Wollman. (Introduced by W. deW. Andrus.)
From the Department of Surgery, Cornell University Medical College, and The Sloan-Kettering Institute for Cancer Research, New York City.

A considerable literature exists on the effects of irradiating tissue cultures *in vitro* covering studies which have been made on the effect of radiation on mitosis, cell migration, cell morphology and viability.[1,2] In these investigations, generally, active growing cultures of various types of tissues have been used, but no work as far as we know has been done on the effects of radiation on freshly explanted normal adult tissues. When a piece of such normal adult tissue is removed from an animal and cultivated *in vitro*, a period of time called the latent period elapses before any cells appear at the margin of the explant.[3] Our present studies were undertaken to determine the effects of various doses of X-ray on the duration of the latent period.

Experimental method. Adult chicken cardiac muscle was cut into small fragments (approximately 2 mm^3). Groups of from 4 to 9 of these fragments were placed in Carrel flasks containing 0.5 cc of chicken plasma, 1 cc of Tyrode's solution and one drop of embryonic extract to facilitate formation of a coagulum. These flasks were then placed in an incubator at 38°C for a period of 20 minutes, exposed for 5 minutes to 5°C (the short 5°C exposures occurred during transport of cultures), after which they were again kept at 38°C for 20 minutes and then irradiated. During the entire period of irradiation (about 10 minutes) experimental as well as the control flasks were kept at 25°C. Following irradiation, the flasks were again subjected to 5°C for 5 minutes, after which they were kept in the incubator at 38°C. All flasks were removed from the incubator for 15 minutes every 6 hours in order to observe the tissue fragments under the microscope and note the appearance of the first cells. Each curve in Fig. 1 represents the collected observations on 30 to 40 pieces of tissue.

The source of the X-rays was a 180 Kv tungsten target experimental X-ray machine, running at 25 milliamperes and using a filtration of 0.5 mm Cu + 1.65 mm Al; the half value layer of transmitted X-rays was 0.8 mm

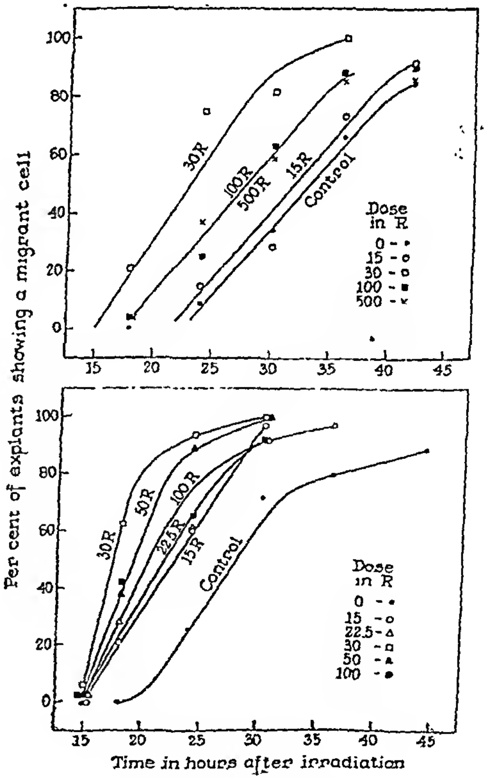

Fig. 1.

Curves showing how migration of cells from explants of adult chicken heart depends on the time after irradiation for various doses of X-rays.

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* This work was supported by a grant furnished by the Committee on Growth of the National Research Council.

[1] Spear, F. G., *Brit. J. Radiol.*, 1935, 8, 68, 280.

[2] Lea, D. E., Action of Radiations on Living Cells, Pub. *Cambridge-Macmillan*, 1947.

[3] Hoffman, R. S., Goldschmidt, J., and Doljanski, L., *Growth*, 1937, 1, 228.

TABLE I.
Response of Chicks to Supplementation with Vitamin B_{12}.

Group No.	Supplement	No. of chicks surviving	G gain during 14-day test period	Hemoglobin, g
1	None	7	105	8.33
2	3% condensed fish solubles	10	190	8.52
3	Vit. B_{12} 0.75 γ/100 g ration	10	152	8.42
4	" " 1.5 γ/100 g "	9	193	8.98
5	Liver extr. (Lilly, reticulogen 20 U.S.P. units/cc), 0.5 U.S.P. unit bird/day*	10	191	8.52
6	Vit. B_{12}, 0.01/γ/bird/day*	10	126	8.84
7	" " 0.1 γ/ " "	10	177	8.81
8	" " 0.5 γ/ " "	10	184	8.60

* Injected intramuscularly.

yellow corn, wheat, soybean oil meal, fish meal and meat scrap. The experimental groups were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The basal ration contained ground yellow corn 35, soybean oil meal 28, wheat bran 10, wheat middlings 10, dehydrated alfalfa meal 5, vitamin test casein 7.5, limestone grit 2.0, steamed bonemeal 1.5, iodized salt 0.5, fish oil (2000A-400D) 0.5, $MnSO_4 \cdot H_2O$ 0.025, iodinated casein (Protamone, 3.07% thyroxine) 0.05 g; thiamine 0.3, riboflavin 0.6, niacin 5.0, calcium pantothenate 2.0, pyridoxine 0.4, p-aminobenzoic acid 10, choline chloride 150, inositol 100, folic acid 0.05, biotin 0.02, menadione 0.05, and α-tocopherol 0.3 mg.

The chicks were wing-banded and weighed when one day old and placed on the basal ration. Individual weights were recorded at weekly intervals. Selection of the birds to be used for the experiment was made at the end of a 2 week depletion period. Mortality during the first 2 weeks was 9%. In order to limit individual variation, two criteria for selection were used: (1) weight (chicks below 60 or above 105 were discarded) and (2) rate of gain (the % gain during the second week was calculated for each bird and those within the range of 33 to 59% were kept). Of the chicks surviving the 2 week depletion period 17% were discarded on the basis of weight and 16% were discarded on the basis of % gain. The selected chicks were distributed (10 per group) in such a way that the average weights of the groups fell between 85 and 88 g and the average % gain ranged from 47 to 48%.

Supplements were added on the 15th day and continued for a 14 day period. Supplementation of the basal ration with 3% condensed fish solubles was made at the expense of corn. The vitamin B_{12} solution (25γ/ml) supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, N. J., was diluted with distilled water and thoroughly mixed into the basal ration by a mechanical mixer to provide 0.75 γ and 1.5 γ of vitamin B_{12} per 100 g. Injections of vitamin B_{12} and liver extract were made into the pectoral muscle using a 1 cc tuberculin hypodermic syringe calibrated in 0.01 cc. Dilutions of vitamin B_{12} with distilled water were such that 0.2 cc injected every other day provided the dosages listed in Table I. Hemoglobin determinations were made on all chicks on the final day of the test period.

Results. The results are presented in Table I. The positive control for groups given vitamin B_{12} in the basal ration was a group receiving a supplement of 3% condensed fish solubles. Groups receiving vitamin B_{12} by injection were compared with a group injected with a liver extract (Lilly, reticulogen, 20 U.S.P. units per cc), at a dosage of 0.5 U.S.P. units per bird per day. The two positive controls showed average weight differences from the negative control of 85 g (condensed fish solubles) and 86 g (reticulogen).

Pure vitamin B_{12} at a level of 0.75 γ per 100 g of ration caused an average increase in weight of 47 g over the unsupplemented group. At a level of 1.5 γ of vitamin B_{12} per 100 g of ration the average weight in-tion with the observation of Kaplan[6] who found that irradiation of a lymphosarcoma *in vivo* with 400 r significantly increased the number of metastases over the number occurring in unirradiated controls.

Summary. Evidence is presented that irradiation with small doses of X-rays decreases the time—latent period—required for a migrating cell to appear at the margin of a fresh explant of normal adult chicken heart. The maximum shortening of the latent period occurs with a dose of about 30 r. With larger doses of X-rays the latent period increases again, although a dose of the order of 1,000 r is necessary before the latent period is as long as that of the controls.

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[6] Personal communication.

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Activity of Vitamin B_{12} in the Growth of Chicks.*

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The requirement of the growing animal for certain dietary essentials can be increased by inducing a hyperthyroid condition. Ershoff,[1] and Betheil *et al.*[2] observed that liver effectively counteracted a thyrotoxicity in rats which were fed desiccated thyroid. Robblee *et al.*[3] found that the addition to the basal ration of either desiccated thyroid or iodinated casein improved the effective assay range for an unidentified chick growth factor. Work has been in progress[4] for some time on the properties of the growth stimulating components of condensed fish solubles. Fractionation procedures indicated similarities between the chick factor and the material in liver which is active in the treatment of pernicious anemia. Injectable liver extracts were found to be active in promoting the growth of chicks fed rations which were complete in the known growth essentials.[5]

Rickes *et al.*[6] reported the isolation from liver of a red crystalline compound termed vitamin B_{12} which was active in microgram quantities for the remission of pernicious anemia in relapse. More recently Ott *et al.*[7] showed that crystalline vitamin B_{12} had "animal protein factor" activity for the chick. Our data confirm this observation and indicate that vitamin B_{12} is highly active in stimulating the growth of the hyperthyroid chick.

Method. The chicks (New Hampshire ♂♂ x Single Comb White Leghorn ♀♀) were the progeny of hens fed diet B_1 described previously[8] in which protein was provided by

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by funds supplied by the Commercial Solvents Corporation, Terre Haute, Ind., and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

[2] Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

[3] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 400.

[4] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Biol. Chem.*, 1948, **173**, 117.

[5] Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 419.

[6] Rickes, E. L., Brink, N. G., Konuiszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

[7] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, 1948, **174**, 1047.

[8] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.*, 1948, **27**, 442.

TABLE I.
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6	Vit. B_{12}, 0.01/γ/bird/day*	10	126	8.84
7	" " 0.1 γ/ " "	10	177	8.81
8	" " 0.5 γ/ " "	10	184	8.60

* Injected intramuscularly.

yellow corn, wheat, soybean oil meal, fish meal and meat scrap. The experimental groups were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum.* The basal ration contained ground yellow corn 35, soybean oil meal 28, wheat bran 10, wheat middlings 10, dehydrated alfalfa meal 5, vitamin test casein 7.5, limestone grit 2.0, steamed bonemeal 1.5, iodized salt 0.5, fish oil (2000A-400D) 0.5, $MnSO_4 \cdot H_2O$ 0.025, iodinated casein (Protamone, 3.07% thyroxine) 0.05 g; thiamine 0.3, riboflavin 0.6, niacin 5.0, calcium pantothenate 2.0, pyridoxine 0.4, p-aminobenzoic acid 10, choline chloride 150, inositol 100, folic acid 0.05, biotin 0.02, menadione 0.05, and α-tocopherol 0.3 mg.

The chicks were wing-banded and weighed when one day old and placed on the basal ration. Individual weights were recorded at weekly intervals. Selection of the birds to be used for the experiment was made at the end of a 2 week depletion period. Mortality during the first 2 weeks was 9%. In order to limit individual variation, two criteria for selection were used: (1) weight (chicks below 60 or above 105 were discarded) and (2) rate of gain (the % gain during the second week was calculated for each bird and those within the range of 33 to 59% were kept). Of the chicks surviving the 2 week depletion period 17% were discarded on the basis of weight and 16% were discarded on the basis of % gain. The selected chicks were distributed (10 per group) in such a way that the average weights of the groups fell between 85 and 88 g and the average % gain ranged from 47 to 48%.

Supplements were added on the 15th day and continued for a 14 day period. Supplementation of the basal ration with 3% condensed fish solubles was made at the expense of corn. The vitamin B_{12} solution (25γ/ml) supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, N. J., was diluted with distilled water and thoroughly mixed into the basal ration by a mechanical mixer to provide 0.75 γ and 1.5 γ of vitamin B_{12} per 100 g. Injections of vitamin B_{12} and liver extract were made into the pectoral muscle using a 1 cc tuberculin hypodermic syringe calibrated in 0.01 cc. Dilutions of vitamin B_{12} with distilled water were such that 0.2 cc injected every other day provided the dosages listed in Table I. Hemoglobin determinations were made on all chicks on the final day of the test period.

Results. The results are presented in Table I. The positive control for groups given vitamin B_{12} in the basal ration was a group receiving a supplement of 3% condensed fish solubles. Groups receiving vitamin B_{12} by injection were compared with a group injected with a liver extract (Lilly, reticulogen, 20 U.S.P. units per cc), at a dosage of 0.5 U.S.P. units per bird per day. The two positive controls showed average weight differences from the negative control of 85 g (condensed fish solubles) and 86 g (reticulogen).

Pure vitamin B_{12} at a level of 0.75 γ per 100 g of ration caused an average increase in weight of 47 g over the unsupplemented group. At a level of 1.5 γ of vitamin B_{12} per 100 g of ration the average weight increase was 88 g more than the negative control.

Vitamin B_{12} was injected at 3 dosage levels: 0.01 γ, 0.1 γ and 0.5 γ per bird per day. The growth response was 21 g at the 0.01 γ level, 72 g at the 0.1 γ level and 79 g at the 0.5 γ level.

No significant difference was noted in the average hemoglobin values of the experimental groups.

Discussion. It is apparent that pure vitamin B_{12} is able to replace the growth stimulating properties of condensed fish solubles or injectable liver extract under the experimental conditions described. It is a reasonable assumption that the properties of these complex materials which have been under study by means of fractionation procedures and chick growth assay, are due to the occurrence of vitamin B_{12}.

The thyrotoxic condition in chicks induced by feeding a ration containing 0.05% iodinated casein is characterized by the hyperexcitability of the birds and depressed rate of gain in weight. The growth response to supplementation is not dependent upon a hyperthyroid condition but the use of a basal ration containing iodinated casein increases the magnitude of the growth response.[3] The role of vitamin B_{12} in counteracting a thyrotoxic condition will require further detailed investigation.

When vitamin B_{12} was fed at a level of 0.75 γ per 100 g of ration the growth response was highly significant and the rate of gain was approximately half maximal under the conditions of this assay. At a level of 1.5 γ vitamin B_{12} per 100 g of ration the growth response compared closely with that of a group receiving 3% condensed fish solubles. This material is recognized as a potent source of the animal protein factor and the rate of gain induced by a 3% level of condensed fish solubles was observed in previous studies to be close to maximum.

Injection of vitamin B_{12} at a level of 0.01 γ per bird per day resulted in a marginal growth response. At a dosage of 0.5 γ per bird per day the response compared closely with that of a group injected with liver extract (Lilly, reticulogen) at a level of 0.5 U.S.P. unit per bird per day. This liver extract was previously observed to be highly effective in stimulating the growth of the hyperthyroid chick.[5] The response observed at a level of 0.1 γ vitamin B_{12} per bird per day was not appreciably lower that that at the 0.5 γ level.

Summary. Vitamin B_{12} administered orally or parenterally completely counteracted a thyrotoxic condition in chicks produced by feeding a basal ration containing 0.05% iodinated casein. A level of 0.75 γ vitamin B_{12} per 100 g of ration resulted in a half maximal growth response. At a level of 1.5 γ vitamin B_{12} per 100 g of ration the growth response after a two week test period was 88 g compared to 86 g for a group supplemented with 3% condensed fish solubles. A response of 72 g resulted from the intramuscular injection of 0.1 γ vitamin B_{12} per bird per day.

Pure vitamin B_{12} can replace the animal protein factor activity of condensed fish solubles and injectable liver preparations.

We are indebted to Merck and Co., Inc., Rahway, N. J., for pure vitamin B_{12} and for crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for synthetic folic acid; to the Cerophyl Laboratories, Inc., Kansas City, Mo., for protamone; and to the Borden Company, New York, for condensed fish solubles.

16818

Lipotropic Activity of Various Compounds Under Standardized Conditions.

M. J. Raymond and C. R. Treadwell.

From the Department of Biochemistry, School of Medicine, George Washington University, Washington, D.C.

A large number of compounds has been tested for lipotropic activity since the demonstration of the action of choline on liver fat.[1] The laboratories carrying out such studies have used various experimental conditions for demonstrating lipotropic activity. It has often been suggested that the lack of uniformity in conditions has hindered correlation of the data obtained by different investigators. In testing the activity of various substances mice,[2] rats,[3] and depancreatized dogs[4] have been used. The stage of the life cycle of the experimental animal has received little consideration. The diets used for producing fatty infiltration of the liver have ranged from essentially fat-free to very high-fat diets, from protein-free to approximately 25% protein diets.[1] The carbohydrate component has been varied in amount and kind, and dietary mixtures of 2 or 3 different proteins have been utilized.[1] Incomplete vitamin mixtures have been used in special cases.[1] In view of the varying conditions under which different compounds have been reported to be lipotropically active, it seemed important to study a representative group of these compounds under standardized conditions. The substances chosen for study have previously[1] been shown to be lipotropically active.

Experimental. The experimental conditions and techniques were those used in this laboratory in previous studies of lipotropism.[5] Male rats of the Carworth strain weighing approximately 170 g were used. The basal diet contained 15.4% casein, 3.2 % arachin, 5% salt mixture, 2% cellu flour, 34.4% glucose, and 40% lard. This purified choline-free diet has been used extensively in this laboratory in studies of growth and lipotropism. It supplies 500 mg of methionine and 100 mg of cystine per 100 g of diet. Animals of the Carworth strain and of this body weight grew at a slightly slower rate on the basal diet than the strain maintained in this laboratory. Also supplementary choline produces a stimulation of the growth rate in these animals while it fails to do so in our strain.[5] The experimental period was 21 days. All rats received orally 0.1 cc of U.S.P. XI cod liver oil and 0.1 cc of a solution containing 25 γ of thiamine, 20 γ of riboflavin, 100 γ of calcium pantothenate, 100 γ of nicotinic acid and 20 γ of pyridoxine per day. The diets and distilled water were available *ad libitum.* The food intake was determined daily and the weight changes recorded 3 times weekly. The livers were removed from the animals under pentobarbital anesthesia and analyzed for total lipids.[6] In the table the weight changes are recorded as percentage change from the initial weight and liver fat is given in terms of g of fat per 100 g of moist liver, and per 100 g of body weight.

Compounds. With one exception the compounds tested were introduced into the basal diet at the expense of the glucose. The dimethyl sulfide was dissolved in corn oil and injected intraperitoneally each day in an amount of 7 mg per rat. The choline, inositol, betaine, and dimethyl sulfide were commercial products of acceptable purity. The S-methyl-

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[1] McHenry, E. W., and Patterson, J. H., *Physiol. Rev.*, 1944, **24**, 128; Best, C. H., and Lucus, C. C., in Harris, R. S., and Thiamann, K. V., Vitamins and Hormones, New York, 1943, **1**; Bach, S. J., *Biol. Rev.*, 1945, **20**, 158.

[2] Eckstein, H. C., and Singal, S. A., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 512.

[3] Best, C. H., and Ridont, J. H., *Ann. Rev. Biochem.*, Stanford University, 1938, **7**, 349.

[4] Van Prohaska, J., Dragstedt, L. R., and Harms, H. P., *Am. J. Physiol.*, 1936, **117**, 166.

[5] Treadwell, C. R., *J. Biol. Chem.*, 1945, **160**, 601.

[6] Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, 1937, **121**, 479.

TABLE I.
Lipotropic Activity of Various Compounds Under Standardized Conditions.

Dietary supplement	%	No. of rats	Change in wt,† %	Food intake per day,† g	Liver lipids per 100 g	
					Moist liver,† g	Body wt,† g
Basal		10	17.6 ± 2.8	9.1 ± .5	19.1 ± 2.5	1.02 ± .20
Lipociac	0.1	8	17.3 ± 1.7	10.5 ± .4	20.5 ± 2.1	1.06 ± .18
S-ethylcysteine	0.1	7	24.3 ± 2.5	10.2 ± .3	18.6 ± 1.4	.97 ± .11
Cystine betaine	0.1	4	26.4 ± 5.4	11.0 ± .2	17.4 ± 2.8	.92 ± .23
S-methylcysteine	0.1	8	19.8 ± 1.9	10.5 ± .3	17.3 ± 1.0	.91 ± .12
Inositol	0.1	7	26.4 ± 2.9	9.4 ± .2	16.0 ± 1.8	.73 ± .14
Dimethyl sulfide*		8	24.2 ± 3.6	9.7 ± .4	14.8 ± 2.1	.71 ± .20
S-methyl-isothiourea	0.2	7	0.9 ± 3.4	7.0 ± .3	11.5 ± 0.5	.62 ± .04
Betaine	0.1	7	27.9 ± 1.8	8.8 ± .4	12.8 ± 1.1	.52 ± .06
Choline	0.05	8	30.9 ± 5.7	10.3 ± .6	12.3 ± 1.2	.49 ± .07
Choline	0.1	7	30.2 ± 2.5	9.8 ± .5	11.0 ± 1.8	.41 ± .04
Triethyl-choline	0.1	8	23.4 ± 3.6	10.7 ± .4	10.2 ± 1.2	.41 ± .06
Lipociac	1.0	7	39.1 ± 1.9	12.2 ± .3	8.5 ± 0.4	.30 ± .01

* See text

† Including the standard error of the mean calculated as follows: $\sqrt{\Sigma D^2/N-1}/\sqrt{N}$

isothiourea sulfate,[7] cystine betaine,[8] S-methylcysteine,[9] S-ethylcysteine,[9] and triethylcholine[10] were synthesized in this laboratory and shown by analysis to be of satisfactory purity. The lipociac was prepared from fresh pork pancreas according to the directions of Clark, Eilert and Dragstedt.[11] It contained 9.77% nitrogen, 1.03% methionine, and .92% cystine. The material gave a negative test for choline with ammonium reineckate both before and after hydrolysis.

Results. The results are shown in Table I. They are arranged in order of decreasing liver fat per 100 g body weight. The choline-containing diets were included for comparative purposes. The lipociac (0.1%) and S-ethylcysteine did not influence the level of the liver fat while the S-methylcysteine and the cystine betaine gave small decreases of questionable significance. Inositol, dimethyl sulfide, and S-methylisothiourea sulfate were intermediate in effect. Choline, betaine, triethylcholine, and lipociac (1%) produced the greatest lowering of the liver fat. The amounts of the substances to be incorporated in the diets were chosen to give a submaximum effect so as to eliminate possible side effects of quantities in excess of that utilized in lipotropism. With none of the experimental diets was the liver fat within the normal range (0.15-0.30 g per 100 g body weight) except that containing 1% lipociac. The results with lipociac at the 1% dietary level are especially interesting. Its lipotropic activity was as great or greater than 0.1% choline and in addition it gave the greatest stimulation of growth. The 1% lipociac supplied 1.26 mg of methionine and 1.12 mg of cystine per rat per day. It has been shown that methionine is approximately 1/5 as active as choline in decreasing the liver fat,[1] and the animals receiving choline (0.1%) in this experiment were ingesting 9.8 mg per day which produced a liver fat of 0.41 g per 100 g body weight. Thus it seems quite unlikely that the methionine content of the lipociac was sufficient to account for the lipotropic effect or the stimulation of growth. Moreover, in order to explain the lipotropic activity of the lipociac on the basis of choline as a contaminant, the lipociac would have had to contain approximately 10% choline which certainly would have been detected by the reineckate procedure. These effects of lipociac are being further investigated. The marked depression of the growth rate by S-methylisothiourea sulfate is also of interest and suggests that further investigation with this compound might be profitable. It is possible that the effect of S-methylisothiourea sulfate on growth is due to the phenomenon of metabolic antagonism.

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[7] Shildneck, P. R., and Windus, W., Organic Synthesis, New York, 1943, Coll., **2**, 411.

[8] Schubert, M. P., *J. Biol. Chem.*, 1935, **111**, 671.

[9] du Vigneaud, V., Loring, H. S., and Craft, H. A., *J. Biol. Chem.*, 1934, **105**, 481.

[10] Channon, H. J., and Smith, J. H. B., *Biochem. J.*, 1936, **30**, 115.

[11] Clark, D. E., Eilert, M. L., and Dragstedt, L. R., *Am. J. Physiol.*, 1945, **144**, 620.

Summary. A representative group of substances, previously reported to be lipotropically active, have been tested under standardized conditions. Under the present experimental conditions and in the amounts fed, S-ethylcysteine did not decrease the level of the liver lipids, cystine betaine and S-methylcysteine exhibited a slight activity of questionable significance, inositol, dimethyl sulfide and S-methylisothiourea sulfate were of intermediate activity, and betaine and triethylcholine had approximately the activity of choline. Lipociac, at the 1% dietary level, was highly active lipotropically and stimulated growth to a greater degree than any of the other compounds tested. S-methylisothiourea sulfate depressed the growth rate.

16819

Effects of Temperature and Ultraviolet Light on Experimental Polyarthritis of Rats.*

HELEN B. TRIPI, GRACE M. GARDNER, AND WILLIAM C. KUZELL.†
From the Department of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco 15, Calif.

The experimental polyarthritis produced by the L-4 strain of pleuropneumonia-like microbes in rats is not strictly comparable to rheumatoid arthritis in man. However, because it responds to gold therapy similarly to rheumatoid arthritis, we consider it a method for studies of factors which might influence the arthritic processes and for making chemotherapeutic trials in animals.

In this study attempts were made to determine whether changes in environmental temperature or in ultraviolet irradiation would alter the course of the disease. This is of importance since, in man, it has commonly been supposed that changes in temperature and ultraviolet irradiation would alter the course of rheumatoid arthritis and the incidence of rheumatic fever. Therefore, we decided to evaluate the effects of these environmental changes with the object of increasing basic knowledge regarding the natural history of the disease in the rat.

Exposure. Thirty male and 30 female albino rats weighing 100 g were placed in an outdoor shelter with open screen sides which protected them from wind, rain and sunshine but not from changes in temperature. The nocturnal temperature ranged from 30 to 45°F, mean 37°F; the diurnal temperature ranged from 46 to 84°F, mean 54°F, the overall range being 30 to 84°F, mean 45°F. The temperature records were made on an automatic thermograph. The average relative humidity was 86% at 5 A.M., 64% at noon, and 70% at 5 P.M.‡ The diet used was Purina Dog Checkers (compressed pellets containing protein 21%; fat 4%; fibre 6%; nitrogen-free extract 46%; and ash 9%). All animals were allowed free access to water. For one week prior to inoculation with pleuropneumonia-like organisms (P.L.O.§) the animals were subjected to exposure. They were then given intraperitoneally 2 cc of a

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† With the technical assistance of Selig A. Gellert and Pelagio S. Tabar.

‡ Official figures supplied by U. S. Weather Bureau, San Francisco.

§ Abbreviation used throughout to designate L_4 strain of pleuropneumonia-like organisms.

TABLE I.
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† Including the standard error of the mean calculated as follows: $\sqrt{\Sigma D^2/N-1}/\sqrt{N}$

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TABLE I.
Influence of Temperature and Ultraviolet on Experimental Polyarthritis of Rats.

	Controls* (70°F)		Exposure† (30 to 84°F)		Ultraviolet‡		Heat§ (102°F)
	Female	Male	Female	Male	Female	Male	Female
Composite arthrogram score	2.9	4.2	2.5	2.3	3.3	1.9	2.6
% showing gross joint involvement	95	100	80	65	75	60	88
% dead within 4 days	5	0	15	10	10	5	0
% total deaths	15	10	35	40	50	45	12
% dead or infected	100	100	95	70	100	90	88
% showing no symptoms	0	0	5	30	0	10	12

* Total No. of rats, 40.

† Exposure controls: 3 deaths were probably due to exposure, leaving 85% survived; total No. of uninoculated rats, 20; No. of inoculated rats exposed to cold, 60.

‡ Ultraviolet controls: No arthritis developed. One animal (male) died of undetermined cause; total uninoculated rats, 20; total inoculated and irradiated rats, 60.

§ Total, 8 rats.

scores than the inoculated controls kept at 70°F and not subjected to additional ultraviolet light. Only 75% of the females and 60% of males developed arthritis as compared with 95% and 100%, respectively, among the inoculated controls. The death rate was higher than that of the inoculated controls, being 50% for females and 45% for males as compared with 15% and 10%, respectively.

Conclusion. In the experimental polyarthritis of rats produced by the L_4 strain of pleuropneumonia-like organisms exposure to cold or increased ultraviolet light under the conditions used caused increased mortality but resulted in a smaller incidence and severity (females only) of arthritis; whereas, increased heat caused little deviation in mortality and arthritis involvement from the inoculated controls.

16820

Protein Intake and Leishmaniasis in the Hamster.*

ALBERT L. RITTERSON AND LESLIE A. STAUBER. (Introduced by J. B. Allison.)
From the Bureau of Biological Research and the Department of Zoology, Rutgers University, New Brunswick, N. J.

It has already been shown that the course of a parasitic disease may be altered by the level of protein intake of the host,[1-2] low levels of intake decreasing host resistance.

Leishmaniasis in the hamster was chosen for the present study because *Leishmania donovani*, the causative agent of kala-azar, a highly fatal human disease is an intracellular parasite living and reproducing in the vertebrate host within the cells of the so-called reticulo-endothelial or lymphoid-macrophage system.

Blockade of the R-E system,[3] *e.g.* with india ink, has been shown to result in a lowering of the resistance of the animal involved; but, to be effective, repeated injections of ink are necessary. The growth and reproduction of leishmania in the R-E cells is believed to constitute a functional blockade of this

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[2] Seeler, A. O., and Ott, W. H., *J. Nat. Malaria Soc.*, 1946, **5**, 123.

[3] Jaffe, R. H., *Physiol. Rev.*, 1931, **11**, 277.

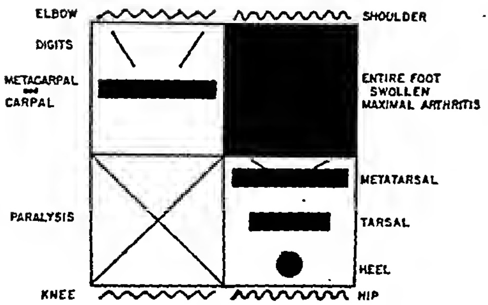

FIG. 1.
Athrogram for recording the extent of joint involvement in experimental polyarthritis of rats.

24-hour broth culture of P.L.O. The animals were examined for gross evidence of arthritis at the end of 4 days, daily for the next week, and then weekly for the following 6 months. The extent of arthritis was recorded on an arthrogram (Fig. 1) which was a modication of Sabin's arthogram.[1] For scoring purposes numerical values were assigned corresponding to the extent of arthritic involvement. The score values ranged from 0 to 4 for each anterior extremity and from 0 to 5 for each posterior extremity; thus the arthrogram score for any individual rat lay within a range of 0 to 18, 0 indicating no demonstrable arthritis and 18 indicating all 4 extremities were involved to a maximum degree. Single composite arthrogram scores were calculated for each animal and group of animals to represent the maximal arthritic involvement during the 6-month period of observation. Ten animals of each sex were uninoculated and kept as controls. In addition to the 20 controls, 20 males and 20 females were inoculated and kept in the animal room as controls where the average daytime temperature was 70°F and the night temperature 58°F.

The rats exposed to cold responded by developing a heavy growth of fur; however, 3 of the uninoculated controls died presumably from the effects of exposure. In the inoculated group, 35% of the females and 40% of the males died, while only 80% and 65%, respectively, showed arthritis. There appeared to be no loss of body weight attributable to the change in environment. The severity of arthritis according to the composite arthrogram score was slightly less than that of the inoculated animals kept at 70°F and the uninoculated controls subjected to cold did not develop arthritis, but only 85% survived.

Heat. Eight female albino rats weighing 100 g were placed in a heated cabinet with an average temperature of 102°F for 24 hours with little variation. The animals were inoculated as described above with P.L.O. They had the same diet but were given thiamine hydrochloride in the drinking water, since rats kept at high temperatures rapidly develop thiamine deficiency. The animals kept at 70°F served as controls for this group.

Immediately after the animals were placed in the heated cupboard they ate less for 3 or 4 days and thereafter resumed normal food consumption. After 6 months of heating these animals had gained normally in body weight, and their tails became longer than those of the controls. Ten heated males died before the scheduled inoculation. Eight heated females, which were inoculated, showed composite arthrogram scores slightly less than for the inoculated controls kept at 70°F. Only 88% of these females developed arthritis as compared with 95% for the controls. The total mortality of these females was only 12% as compared with 15% for the inoculated controls, 35% for those subjected to exposure, and 50% for the females subjected to ultraviolet light.

Ultraviolet Irradiation. Thirty male and 30 female albino rats were exposed for 6 months to constant ultraviolet irradiation supplied by a Westinghouse Type S-1 ultraviolet lamp at a distance of 5 feet. The average temperature remained constant at 82°F. Ten animals of each sex were used as uninoculated controls.

A few of the irradiated rats developed a mild conjunctivitis, but there was no increase in body weight or food consumption. Among the uninoculated controls subjected to ultraviolet light one animal died of undetermined cause and none developed arthritis. Among the inoculated animals the females had higher and the males lower composite arthrogram

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[1] Sabin, A. B., and Warren, J., *J. Bact.*, 1940, **40**, 823.

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system with increasing numbers of parasites matching the compensatory hyperplasia of the R-E cells.

The problem under study was to learn the effect of such an endogenous blockade of the R-E cells on animals fed diets deficient in protein as well as on animals fed diets containing excess of protein.

Materials and methods. The Syrian hamster, *Cricetus auratus*, was chosen as the host because of its proven susceptibility to *L. donovani* and because the course of leishmaniasis in it is similar to that seen in man. Infection was obtained by the intraperitoneal inoculation of amounts of a saline suspension of hamster spleen, ground in a Ten Brock tissue grinder, suitable to produce severe infection in 60-100 days. The usual dose was equivalent to 20 mg of a heavily infected spleen. Aseptic precautions were observed in the transfer of the infection. The parasite used was of the Khartoum strain and obtained by us from Dr. A. P. Richardson of the Squibb Institute for Medical Research.

TABLE I.
Composition of the Hamster Diets Used.

Basic Protein (20%)	g
Casein	200
Primex or Crisco	250
Corn Oil	20
Cerelose	200
White Dextrin	257
Salt Mixture[*]	40
Cellu Flour	20
A, D, and E conc.[†]	1
Choline Chloride	2
Wilson's 1:20 liver powder[‡]	10
	1000

To each 1000 g add 0.372 g of vit. B mixture[§] and 0.010 g vit. K.

Low Protein (10%)
Substitute in above:
100 g Casein
357 g Dextrin
High Protein (40%)
Substitute in above:
400 g Casein
57 g Dextrin

[*] Wesson, *Science*, 1932, **75**, 339.

[†] A, D, and E concentrate:

	g
Corn oil	41
A and D conc. (450,000 U. S. P. units of A) (90,000 U. S. P. units of D)	7
Alpha tocopherol	2
	50

[‡] Armour's liver extract (23 ml) used in place of liver powder.

[§] Vit. B mixture:

	mg
Thiamine hydrochloride	200
Riboflavin	400
Pyridoxine HCl	200
Niacin	1000
Calcium pantothenate	1100
Para-aminobenzoic acid	1000
Inositol	5400
	9300

The principal diet used was a modified mouse diet[†] (Table I). Although niacin is apparently not necessary in the diet of the hamster[4] it was included here since animals on protein deficient diets may not have the same requirements as animals on control diets. One or two weeks prior to inoculation all animals were placed on their test diets containing either high (40%), basic (20%) or low (10%) levels of protein. The protein used was casein. The high and basic protein diets allowed immature hamsters to gain weight at approximately a normal rate. All groups of hamsters ate the diet well.

Results. The results obtained may be summarized under the headings of survival, body weight changes, estimated number of parasites and organ weight changes.

Infected animals survived longer when maintained on the high and basic protein diets than on the low protein diet. The animals on the low protein diet (Fig. 1) were all dying when the experiment was terminated while those of the other 2 groups were in fairly good condition. Fig. 1 shows also that the fall in weight of the animals on the low protein diet to a value below their initial weight is a terminal phenomenon. High intake of protein, therefore, partially protects against the progressive emaciation characteristic of the disease.

Although this "protective" effect of the basic and high protein diets seems to be borne out by the relative numbers of parasites in the impression smears made from the

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[†] Details of diet furnished in personal communication through courtesy of Dr. David Bosshardt, Sharp & Dohme Co.

[4] Cooperman, J. M., Waisman, H. A., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 250.

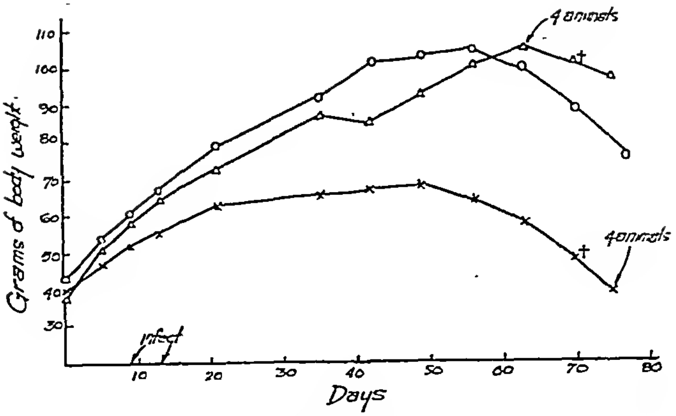

FIG. 1.
Weight curves of immature hamsters, infected with *Leishmania donovani*, fed experimental diets of different protein content: Δ High protein diet (40% casein); ○ Basic protein diet (20%); × Low protein diet (10%).

TABLE II.
Effect of Low, Basic and High Protein Diets on Organs and Organ-weight, Body-weight Ratios; and on the Parasite Counts of Growing Hamsters Infected with *Leishmania donovani*. All figures represent average values for the group. All animals were sacrificed between 75-77 days after being placed on the diets.

Conc. of protein in diet	No. of animals	Body wt (g)	Spleen wt (mg)	Spleen-wt body-wt ratio*	Liver wt (mg)	Liver-wt body-wt ratio*	Estimated No. of parasites
Low (10%)	5	38.1†	209.2†	5.3†	3248†	85.4†	++++
Basic (20%)	5	75.2	488.6	6.5	5143	68.3	++
High (40%)	5	96.2†	840.8†	8.7†	7097†	73.8†	+±

* Ratios expressed as milligrams of organ per gram of body weight.
† Averages represent only 4 animals of the group. One animal died before 75th day.

spleen at necropsy (Table II), when calculated on the basis of the estimated absolute numbers of parasites in the enlarged spleens the differences are much smaller. Indeed, the data seem to suggest that the reproductive rate of the leishmania may be nearly constant on all 3 diets.

The pathology observed was essentially that described by Meleney[5] all heavily-infected animals showing marked splenomegaly and hepatomegaly, both absolute and relative to body weight (Table II and Fig. 2). The liver-weight/body-weight ratio of the normal hamster is approximately 43 mg/g (range of ratio from 40-46 for animals 85-124 g body weight). The ratio in the infected animal usually does not increase quite 100% in value. The normal spleen-weight/body-weight ratio is about 1.4 mg/g for animals weighing from 85-124 g. In the infected

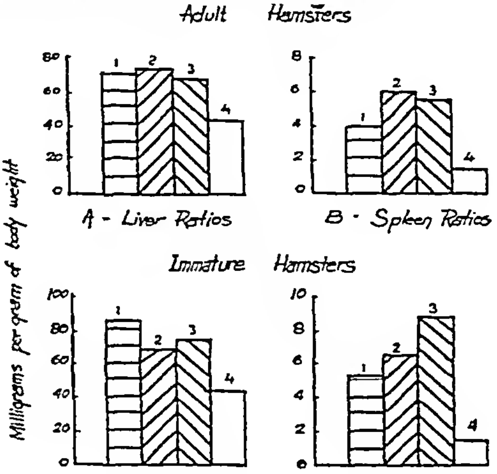

FIG. 2.
Organ weight, body weight ratios for adult and immature, infected and uninfected hamsters fed diets differing in protein content. 1, Low protein diet, infected; 2, Basic protein diet, infected; 3, High protein diet, infected; 4, Stock diet, uninfected.

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[5] Meleney, H. E., *Am. J. Path.*, 1925, 1, 147.

hamster this usually increases more than 300%. The spleen, therefore, is a better index of infection. Except possibly, for the relatively low ratios for the spleens of animals on low protein diets there are probably no significant differences in organ weight ratios under the dietary conditions studied.

Work is now in progress to determine the effect of kala-azar on the liver nitrogen in growing hamsters.

Summary. Protein intake influences the course of leishmaniasis in the hamster, deficient diets leading to earlier emaciation and death. Excess dietary protein seems to favor survival.

16821

Serum Cholinesterase in Some Pathological Conditions.*

M. G. LEVINE AND R. E. HOYT.†‡

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Of the esterases in human blood, the one hydrolyzing acetylcholine into acetic acid and choline has received the most attention in recent years because of the supposed relationship of this enzyme to the physiology of nerve activity. Actually, recent work indicates that there are two enzymes involved. One, the so-called true or specific cholinesterase, is thought to be involved directly in the transmission of nerve impulses, whereas the non-specific or pseudocholinesterase has, as yet, no assigned function.

Since the activity of the latter enzyme is easily measured, and since it is present in a readily available material (serum) in hospital patients, it offers an opportunity to study variations in a basic enzyme system in a number of pathological conditions. Such variations in themselves may be of more importance when the nature and function of the enzyme itself is better understood, nevertheless these variations are a guide to alterations in the metabolism of the organ or organs producing, distributing and disposing of the enzyme.

We have confirmed the findings of those workers who have noted a great spread in the values for pseudocholinesterase in the serum of normal individuals. This may be seen in the summary of normal values in the tables which follow. This spread has confused many observers who have been unable to come to any conclusion as to variations from the normal of serum cholinesterase in numerous pathological conditions. However, such difficulties are obviated by using sufficiently large samples thus permitting statistical analysis.

Of the published information available, there is evidence to indicate a decrease in the value of the enzyme in liver damage[1-6] and in pernicious anemia in relapse.[7,8] We have observed the decrease in cases of liver damage due to a variety of causes. Since this phenomenon has been reported comprehensively in the literature, we are not at this time publishing our data. In three other disease states, either covered not at all or inadequately by previous workers, we have made observations which we are reporting here.

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* Aided by a grant from the Cancer Research Grants Division, U. S. Public Health Service.

† With the technical assistance of Anita A. Suran, who was assisted in part by a fellowship from the California Tuberculosis and Health Association, Medical Research Committee.

‡ We are grateful to Dr. Emil Bogen, Olive View Sanatorium, for assistance in obtaining sera for our tuberculosis studies.

[1] Antopol, W., Tuchman, T., and Schiffren, A., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 46.

[2] Antopol, W., Schiffren, A., and Tuchman, L., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 363.

[3] McArdle, B., *Quart. J. Med.*, 1940, **9**, 107.

[4] Faber, M., *Acta Med. Scand.*, 1943, **114**, 72.

[5] Kunkel, H. G., and Ward, S. M., *J. Exp. Med.*, 1947, **86**, 325.

[6] Wescoe, W. C., Hunt, C. C., Riker, W. F., and Litt, I. C., *Am. J. Physiol.*, 1947, **149**, 549.

[7] Sabine, J. C., *J. Clin. Invest.*, 1940, **19**, 833.

[8] Meyer, L. M., Sawitsky, A., Ritz, N. D., and Fitch, H. M., *J. Lab. and Clin. Med.*, 1948, **33**, 189.

Serum cholinesterase in pregnancy. Butt and co-workers[9] found in 14 women (who were either pregnant at the time or had recently given birth) that the "readings obtained in the course of pregnancy fall within the limits of variation of normal, but with two exceptions they are all below the mean found for normal females." Hall and Lucas[10] and Milhorat[11] in small series found no variation from the normal in pregnancy. Laborit and Morand[12] observed a decrease in serum cholinesterase during labour but an increase during pregnancy and Davis *et al.*[13] found the enzyme to remain normal during pregnancy. Finally, Zeller and associates[14] found no statistical deviation from normal of the enzyme in pregnancy.

In light of the above contradictory findings, we first ran a series of pregnant women at the time of delivery. The following reagents were employed in terms of the final concentration in a total volume of 3 cc:

.1 cc serum
.0017 M. sodium bicarbonate
.0034 M. acetylcholine chloride

The production of acetic acid from acetylcholine was followed at 37° by means of the liberation of carbon dioxide from a bicarbonate-carbonic acid buffer in Warburg vessels. These vessels were gassed at room temperature with a mixture of 95% nitrogen and 5% carbon dioxide. At zero time, the acetylcholine was tipped into the serum and bicarbonate mixture. Readings were taken at 20 and 40 minutes. Since the two are comparable only the latter is given in Table I.

We may calculate from Table I that the difference between the means is 15 μl. CO_2 and the probable error of the difference is 1.81 μl of CO_2 This difference is 8.28 times the probable error (a significant difference, occurring by chance only once in over 15 million trials).

In order to verify the above observations, we repeated the experiment under new conditions. This time the reagents were identical to those used by Mazur and Bodansky[15] who followed essentially the method described by Ammon.[16] In the final reaction mixture of 3 cc, the concentration of the acetylcholine was 0.015 M (pH 7.7). The enzyme was present in .5 cc serum diluted 1:10 in the $NaHCO_3$. The remainder of the procedure is the same as described above. The results for 20 minutes are listed in Table II.

The statistical significance of the data in Table II is evident from the figures on the magnitude of the differences between the means of the pregnant groups and the normal group and the probable error of these differences. If the difference between the means is 10 times the probable error of this difference, the probability of this occurring by chance is 65 billion to one.[17] It is obvious, therefore, that there is a decrease in the serum cholinesterase during pregnancy, and also at the time of delivery.

Serum cholinesterase in tuberculosis. There are a few references to serum cholinesterase in tuberculosis in the medical literature.[18,19] None, however, has a sufficiently large series

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[9] Butt, H. R., Comfort, M. W., Dry, T. V., and Osterberg, A. E., *J. Lab. and Clin. Med.*, 1942, **27**, 649.

[10] Hall, G. E., and Lucas, C. C., *J. Pharm. and Exp. Ther.*, 1937, **59**, 34.

[11] Milhorat, A. T., *J. Clin. Invest.*, 1938, **17**, 649.

[12] Laborit, H., and Morand, P., *Gynecologie et Obstetrique*, 1947, **46**, 298.

[13] Davis, M. E., Si-Feng Yu, E., and Fugo, N. W., *J. Clin. Endocrinology*, 1948, **8**, 666.

[14] Zeller, E. A., Birkhauser, H., Wattenwyl, H. V., and Wenner, R., *Helv. Chim. Acta*, 1941, **24**, 962.

[15] Mazur, A., and Bodansky, O., *J. Biol. Chem.*, 1946, **163**, 261.

[16] Ammon, R., *Arch. ges. Physiol.*, 1930, **233**, 486.

[17] Pearl, R., Medical Biometry and Statistics, Saunders, Phila., 1940.

[18] de Michele, G., *Boll. Soc. Ital. Biol. Sper.*, 1944, **19**, 66.

[19] Crestol, P., Passovant, C., Beneczech, C., and Dutarte, G., *Presse Med.*, 1946, **54**, 557.

TABLE I.

Serum Cholinesterase* in Normal Women and Pregnant Women at the Time of Delivery.

	No. of individuals	Age range	Mean value* in μl CO_2	Stand. Dev.
Normal	67	18-40	90	±17
Pregnant	59	18-40	75	±13

* 40 minutes at 37°C and calculated to 760 mm mercury at 0°C.

TABLE II.

Serum Cholinesterase in Pregnant and Normal Women.

Group	No. of individuals	Age range	Mean value in μl CO_2*	Stand. Dev.	Δ
Prenatal 1-6 months	48	18-40	65.9	±13.9	10.4
” 6-9 ”	38	18-40	64.6	±13.0	10.8
At term	52	18-40	64.3	±19.6	9.5
Normal women	80	18-40	86.7	±19.5	

* 20 minutes at 37°C and calculated to 760 mm mercury at 0°C.

$$\Delta = \frac{\text{Difference between the means}}{\text{Probable error of this difference}} = \sqrt{(P.E._{M1})^2 + (P.E._{M2})^2}$$

TABLE III.

Serum Cholinesterase in Normal People and Individuals with Pulmonary Tuberculosis.

Group	No. of individuals	Mean	± Stand Dev.	Δ
T.B. men	60	82.7	26.5	4.1
Normal men	66	94.2	19.2	
T.B. women	83	80.9	28.5	2.2
Normal women	80	86.7	19.5	

to warrant statistical analysis. We were fortunate in being able to obtain a fairly large number of sera from patients with pulmonary tuberculosis at the Olive View Sanatorium, Olive View, California. A total of 60 men and 83 women were studied. These had been previously classified clinically as follows:

	Total	Advanced	Moderate	Mild
Men	60	50	9	1
Women	83	62	16	5

Serum cholinesterase was determined by the method described above for the second series of pregnant women. The results for tuberculosis are listed in Table III.

From Table III, it may be seen that since Δ is only 4.1 in the comparison of tuberculous and normal men, the probability of this occurring by chance is only 174 to one. In the case of the women, Δ being only *2.2*, the probability of this occurring by chance is 6.25 to 1. We do not feel that these figures are significant. Hence, in our series, we may conclude that pulmonary tuberculosis does not alter the serum cholinesterase values.

Serum cholinesterase in patients with neoplasms. Greenstein[20] has reviewed in a comprehensive fashion variations from normal of enzyme values in tumor bearing hosts. Although the discovery of such variations has not proved to be of diagnostic value, we agree with Greenstein as to their basic importance: “More knowledge is needed on the analytical chemistry of the cancer cell, particularly of its enzymes and coenzymes, of the non-catalytic proteins, of the sugars, nucleic acids, fats and salts—of their analytical distribution, rate of metabolic exchange and source of origin. To an equal extent, the same can be said of normal tissues; the intensive study of these tissues is bound to be helpful in research on tumors if only by stimulation, by the illumination offered by contrasting properties, and by providing new tools and approaches.”

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[20] Greenstein, J. P., Biochemistry of Cancer, Academic Press, New York City, 1947.

TABLE IV.
Serum Cholinesterase in Patients with Malignant Tumors.

Site and type primary tumor	Metastasis	μl CO_2-20 min.
Squamous cell carcinoma hand	+	33.1
'' '' '' tongue	—	63.6
'' '' '' face	—	90.0
'' '' '' larynx	—	58.1
Adeno-carcinoma rectum	—	63.8
'' '' ''	+	47.5
'' '' stomach	—	53.8
'' '' ''	—	50.1
'' '' ''	—	39.2
'' '' ''	+	56.5
'' '' ''	+	39.2
'' '' ''	+	53.7
'' '' prostate	—	33.9
'' '' uterus	—	52.7
Transitional cell carcinoma bladder	—	73.6
'' '' '' ''	—	68.3
'' '' '' ''	—	78.3
'' '' '' ''	—	83.7
Myeloma femur	+	54.2
Reticulum cell sarcoma	—	59.3
	Mean =	62.7
	Standard deviation =	±18.8
	Δ =	8.3

variation in serum cholinesterase in patients with neoplasms. We have studied 26 cases of men and women with a variety of malignant tumors. The reagents and methods were the same as described for pregnant women in Table II.

The Δ value of 8.3 given in Table IV is obtained by comparing the mean for the cancer group with the mean for normal women. This gives a value lower than the true one since the cancer group contains both men and women. However, even with this lower value the probability of the difference between the means occurring by chance is over 15 million to one. We must thus conclude that there is a drop in serum cholinesterase in patients with malignant tumors.

Summary. There is a significant decrease in serum cholinesterase in women in the various stages of pregnancy and in patients with neoplastic disease. There is no decrease in our series in patients with pulmonary tuberculosis.

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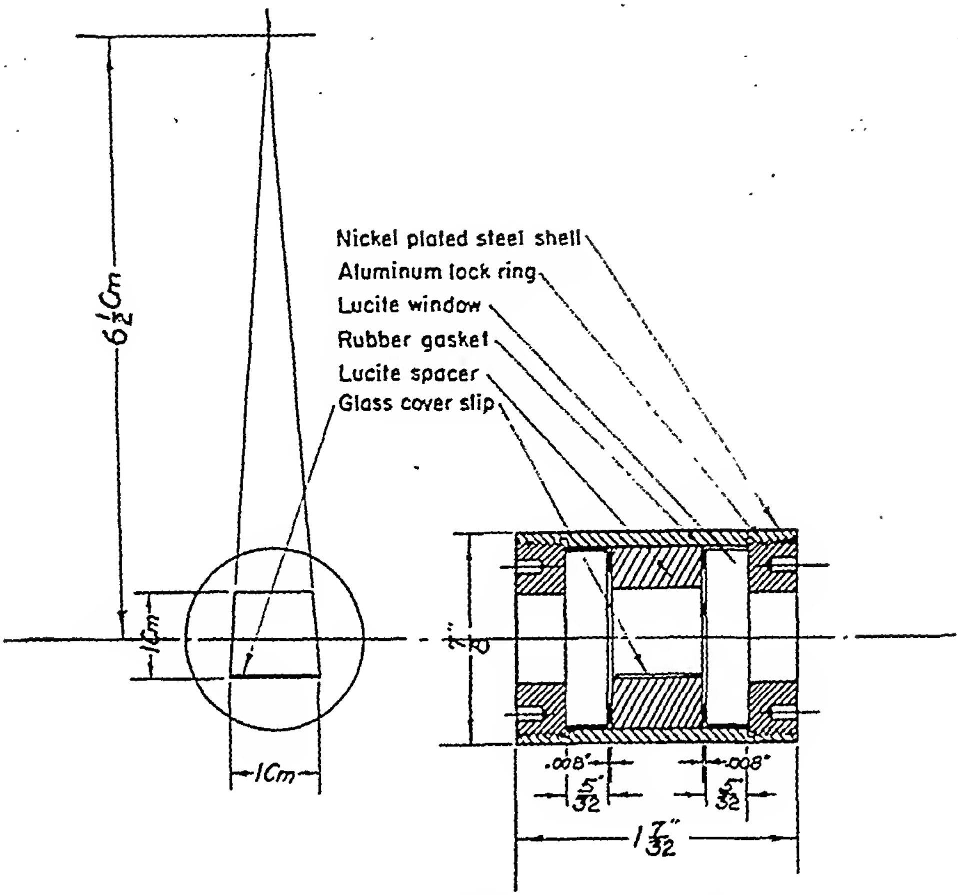

FIG. 1.
Construction details of the rotor cell for the analytical ultracentrifuge. In this cell the virus from about one ml fluid is sedimented onto the collodion-coated glass at the bottom. This glass is then removed; the collodion is stripped off; and the electron microscope is used to count and study the virus deposited upon it.

pension, after which the second window is placed and fastened with the corresponding aluminum ring. The cell is then inserted in the rotor of the analytical ultracentrifuge and, in the present experiment, spun at 16,500 g for 30 minutes; under such conditions the influenza virus particles should sediment, convection free, upon the collodion coating on the glass cover slip. After the run, the cell is opened and the cover slip carefully removed, dipped twice in distilled water to remove the salt and then dried. The film is scratched across with a needle and floated off the glass on a distilled water surface. The film, divided by the scratch, comes off in two pieces, on one of which is placed a standard 200 mesh electron microscope screen. The other piece is caught from below atop another such screen, this being the one with the virus particles on top for use in shadow casting.

Swine influenza virus was obtained from the chorio-allantoic fluid of virus infected chick embryos 4 years ago and formolized for the preparation of vaccines. The procedures for concentration and partial purification of the virus by ultracentrifugation have been described elsewhere.[2] This material was in Ringer solution in a stoppered tube stored in the refrigerator at 2 to 8°C.

Experimental. In the preliminary experi-

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[2] Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, Dorothy, Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

16822

Enumeration of Virus Particles by Electron Micrography.*

D. GORDON SHARP. (Introduced by J. W. Beard.)

From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

In the study of viruses or other particulate materials in aqueous suspension with the electron microscope, it is necessary to secure a thinly spread deposit of the particles on the collodion film. This collodion film is then studied by transmitted electrons either in light or dark field illumination, or the particles on the film may be metal shadowed by the method of Williams and Wyckoff[1] before study. In any case it is desirable that each kind of particle in the suspension be present on each area of the collodion forming one complete field in the electron microscope. It is desirable also that the aggregates, if any, of the particles seen in the microscope should not be produced in the procedure by which the particles were deposited on the film; that is, any aggregation observed should be representative of the state of dispersion of the particles present in the suspension under study. Furthermore, it is highly desirable that the number of particles per unit area of the collodion (per microscope field) be the same from place to place and equal to the number in a known volume of the suspension under study.

At present there is no technic fulfilling these conditions. The usual procedure consists of placing a drop of the virus suspension on the collodion film and leaving it for a few seconds. The fluid is then withdrawn with a fine pipette, and the residual, thin film is left to dry and to deposit its particles on the collodion. Although it is sometimes possible to obtain suspensions of animal viruses in water, this is not the general rule. Most of these materials do not disperse well in water, and other laboratory studies on such viruses are carried out in some suitable buffered salt solution. It is frequently desirable that electron micrographs be obtained with such salt suspensions for study of the state of the virus under these conditions for comparison with other data, those from the ultracentrifuge, for example. However, when the residue of salt solution containing virus dries on the collodion film, changes occur in salt concentration and, probably, in pH as well. The liquid film does not dry evenly, and as a result of a combination of these factors, it is clear that any particular distribution of deposited virus bodies or aggregates among them is likely to bear little resemblance to the state of dispersion of the particles in the suspension from which they came. In this paper[†] a method has been used in the study of formolized swine influenza virus which promises to correct some of the ills described above.

Materials and methods. The method is based on the use of a specially designed cell fitting into the rotor of the air-driven analytical ultracentrifuge. The cell, shown in the drawing of Fig. 1, is similar to the one used for sedimentation velocity studies but with some modifications for the present purpose. As seen in Fig. 1, the cell itself consists of 2 lucite windows held apart by a lucite block in which there is a space of about 1 ml volume with trapezoidal sides coinciding with radii of the rotor. In preparation for filling the cell, one lucite window is put in place with a thin rubber gasket to prevent leakage and locked in with threaded aluminum ring and spanner wrench. A piece of cover slip glass coated with collodion is inserted at the bottom of the cell and the cell filled with the virus sus-

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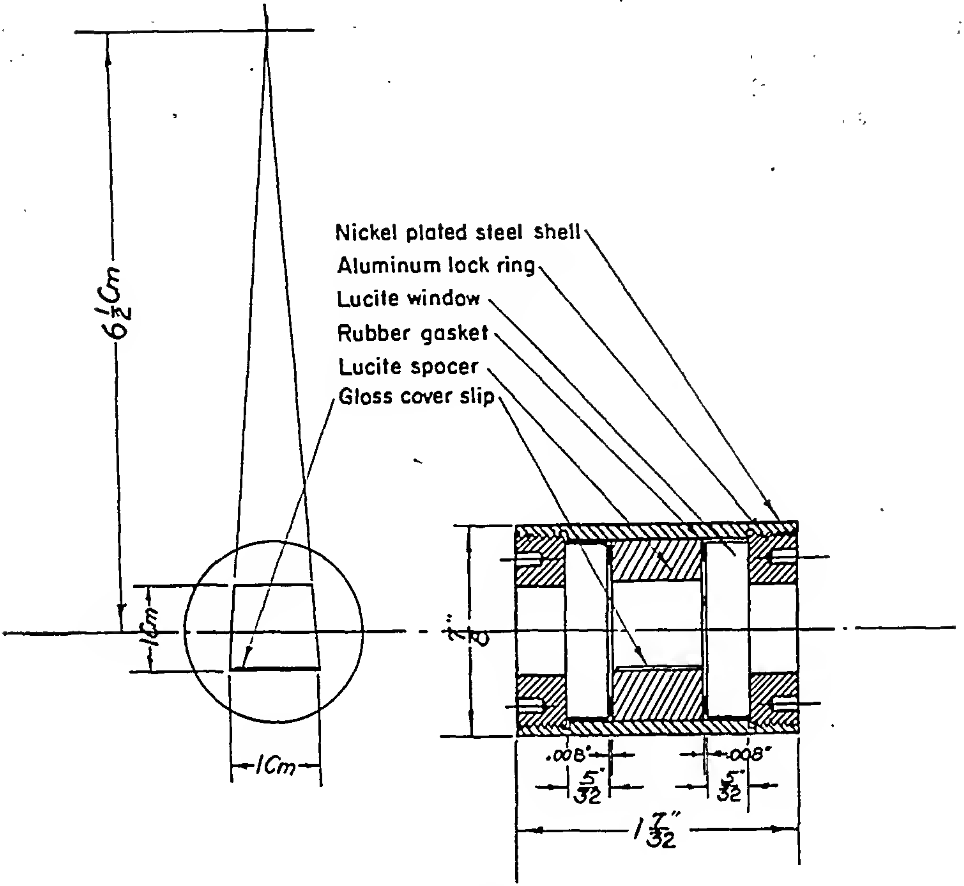
[*] This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

[1] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 265.

[†] This work was reported at the meeting of the Electron Microscope Society of America held in Toronto, September 10, 1948.



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From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

In the study of viruses or other particulate materials in aqueous suspension with the electron microscope, it is necessary to secure a thinly spread deposit of the particles on the collodion film. This collodion film is then studied by transmitted electrons either in light or dark field illumination, or the particles on the film may be metal shadowed by the method of Williams and Wyckoff[1] before study. In any case it is desirable that each kind of particle in the suspension be present on each area of the collodion forming one complete field in the electron microscope. It is desirable also that the aggregates, if any, of the particles seen in the microscope should not be produced in the procedure by which the particles were deposited on the film; that is, any aggregation observed should be representative of the state of dispersion of the particles present in the suspension under study. Furthermore, it is highly desirable that the number of particles per unit area of the collodion (per microscope field) be the same from place to place and equal to the number in a known volume of the suspension under study.

At present there is no technic fulfilling these conditions. The usual procedure consists of placing a drop of the virus suspension on the collodion film and leaving it for a few seconds. The fluid is then withdrawn with a fine pipette, and the residual, thin film is left to dry and to deposit its particles on the collodion. Although it is sometimes possible to obtain suspensions of animal viruses in water, this is not the general rule. Most of these materials do not disperse well in water, and other laboratory studies on such viruses are carried out in some suitable buffered salt solution. It is frequently desirable that electron micrographs be obtained with such salt suspensions for study of the state of the virus under these conditions for comparison with other data, those from the ultracentrifuge, for example. However, when the residue of salt solution containing virus dries on the collodion film, changes occur in salt concentration and, probably, in pH as well. The liquid film does not dry evenly, and as a result of a combination of these factors, it is clear that any particular distribution of deposited virus bodies or aggregates among them is likely to bear little resemblance to the state of dispersion of the particles in the suspension from which they came. In this paper† a method has been used in the study of formolized swine influenza virus which promises to correct some of the ills described above.

Materials and methods. The method is based on the use of a specially designed cell fitting into the rotor of the air-driven analytical ultracentrifuge. The cell, shown in the drawing of Fig. 1, is similar to the one used for sedimentation velocity studies but with some modifications for the present purpose. As seen in Fig. 1, the cell itself consists of 2 lucite windows held apart by a lucite block in which there is a space of about 1 ml volume with trapezoidal sides coinciding with radii of the rotor. In preparation for filling the cell, one lucite window is put in place with a thin rubber gasket to prevent leakage and locked in with threaded aluminum ring and spanner wrench. A piece of cover slip glass coated with collodion is inserted at the bottom of the cell and the cell filled with the virus sus-

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* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

[1] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 265.

† This work was reported at the meeting of the Electron Microscope Society of America held in Toronto, September 10, 1948.

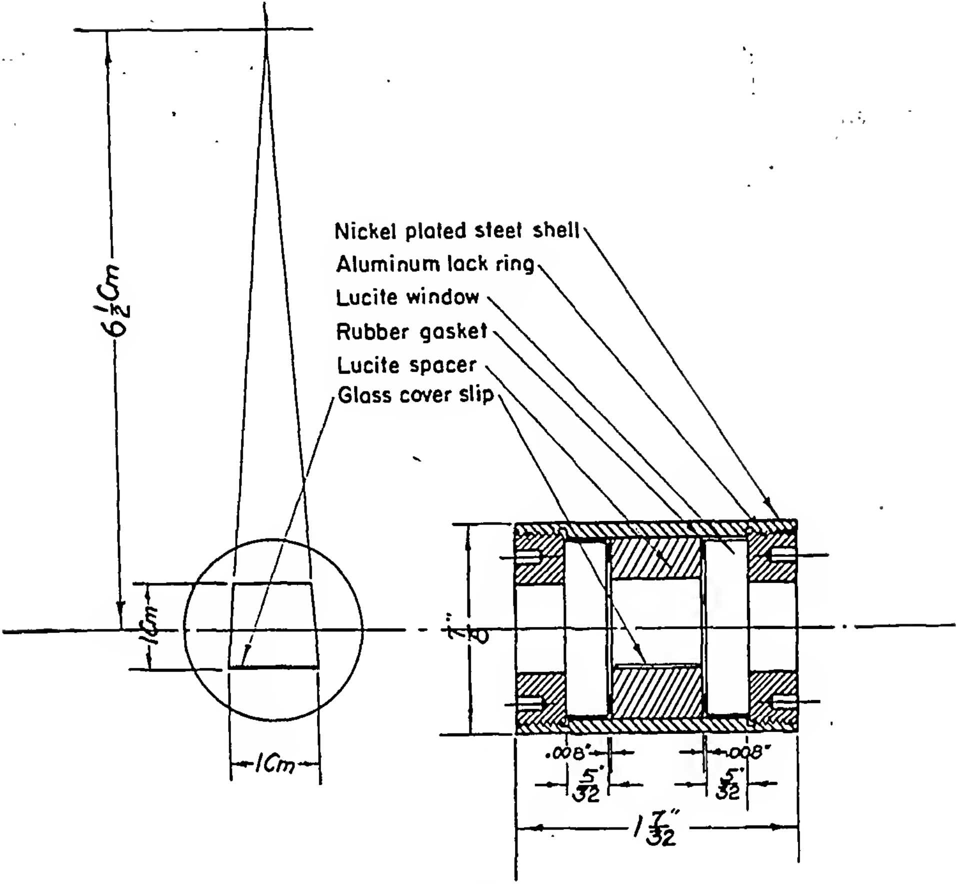

Fig. 1.

Construction details of the rotor cell for the analytical ultracentrifuge. In this cell the virus from about one ml fluid is sedimented onto the collodion-coated glass at the bottom. This glass is then removed; the collodion is stripped off; and the electron microscope is used to count and study the virus deposited upon it.

pension, after which the second window is placed and fastened with the corresponding aluminum ring. The cell is then inserted in the rotor of the analytical ultracentrifuge and, in the present experiment, spun at 16,500 g for 30 minutes; under such conditions the influenza virus particles should sediment, convection free, upon the collodion coating on the glass cover slip. After the run, the cell is opened and the cover slip carefully removed, dipped twice in distilled water to remove the salt and then dried. The film is scratched across with a needle and floated off the glass on a distilled water surface. The film, divided by the scratch, comes off in two pieces, on one of which is placed a standard 200 mesh electron microscope screen. The other piece is caught from below atop another such screen, this being the one with the virus particles on top for use in shadow casting.

Swine influenza virus was obtained from the chorio-allantoic fluid of virus infected chick embryos 4 years ago and formolized for the preparation of vaccines. The procedures for concentration and partial purification of the virus by ultracentrifugation have been described elsewhere.[2] This material was in Ringer solution in a stoppered tube stored in the refrigerator at 2 to 8°C.

Experimental. In the preliminary experiments virus preparations for electron micrography were made in the usual manner. The suspension of formolized virus was diluted one to ten and prepared for the electron microscope by drying a small amount on the collodion film. Such preparations yielded approximately 25 to 100 virus particles per cm^2 in pictures taken at a magnification of 4,500 x. This was about the concentration of virus ordinarily employed for electron microscope study of the purified virus.

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[2] Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, Dorothy, Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

In order, now, to produce pictures of controlled deposition of virus, the procedure departed from the usual methods. A further dilution of the virus suspension 1-100 (total dilution 1-1000) was made with physiological saline solution and this was put into the rotor cell described above and spun for 30 minutes at 16,500 g. Such preparations yielded uniform distribution of virus particles, and pictures were taken with and without shadow casting. The procedure was repeated using 1-2,000, 1-4,000, 1-8,000, 1-16,000 and 1-32,000 dilutions of the same starting material. When the series was finished, repeat runs were made on freshly made 1-4,000, 1-8,000 and 1-16,000 dilutions to check the original data.

All of the electron micrographs were made at a magnification of 4,500 x, and a grid of fine lines one centimeter apart was projected with the original negatives so that the prints would have a counting standard of area independent of photographic enlargement. About a hundred pictures in all were made of various areas of the films produced. The counts per cm^2, as well as the total number of particles counted, and the statistical variation expressed as standard deviation (σ) are shown for the various dilutions and for the repeat runs in Table I. Strips of representative pictures of the chromium shadow-cast preparations are shown in Fig. 2.

Although this procedure is more difficult than simple drying of the material in the usual way, it yields counts agreeing well with the dilution of the starting suspension. Variation in the number of virus particles per unit area is small, and for this reason, one can get a good general impression of the preparation from examination of only a few fields. Furthermore, the counts obtained from repeat runs at a given dilution showed gratifying similarity.

Calculations. Some calculations have been made to determine the number of virus particles to be expected per unit area in these pictures. For this it was necessary to know the weight of virus per ml of the suspension used, the average unhydrated weight of the particles, and the magnification in the picture. The nitrogen content measured on purified swine influenza virus from this laboratory was 9%.[3] For the present work, a sample of the swine influenza vaccine was sedimented at 16,500 g for 30 minutes, and the nitrogen content of the sedimented material obtained by difference between Kjeldahl measurements on the whole material and on the supernate of this run. The resulting value multiplied by the factor 11.1 gave 1.85 mg virus per ml of the starting material. The 1/1,000 dilution thus contained 0.00185 mg virus per ml.

The average particle size, density and water content were taken from previously published data on the freshly purified virus.[4] In order to check the applicability of these data to the formolized virus used in the present experiments, 4 sedimentation velocity runs were made, 2 in physiological saline solution and 2 in a solution of bovine serum albumin in physiological saline (density 1.046). In the first pair of studies the sedimentation rates of the formolized virus corrected to water viscosity at 20°C were 671 and 677 x 10^{-13}, and in the second pair the values were 379 and 377 x 10^{-13}. When these values were plotted against the densities of the respective suspending media, 1.004 and 1.046, it was seen that the formolized virus had a size and density the same as the analogous properties reported for the freshly purified virus.[4] This is taken to mean that no great error will be made in using for particle radius, the value 58.5 mμ; for wet density, 1.100; and for water content by weight, 39.2%.

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[3] Taylor, A. R., *J. Biol. Chem.*, 1944, **153**, 675.

[4] Sharp, D. G., Taylor, A. R., McLean, I. W., *Jr.*, Beard, Dorothy, Beard, J. W., *J. Biol. Chem.*, 1945, **159**, 29.

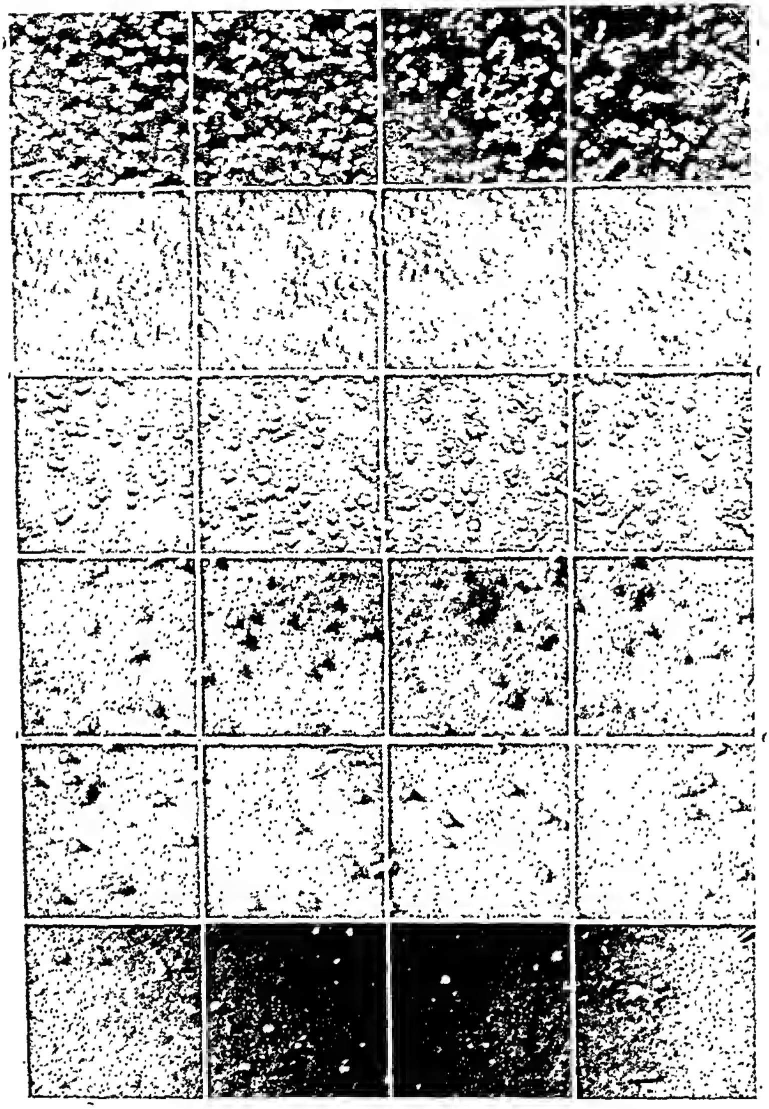

FIG. 2.

Influenza virus deposited upon a collodion membrane by sedimentation in the ultracentrifuge. These strips are representative of pictures taken of deposits from dilutions of 1-1,000, 1-2,000, 1-4,000, 1-8,000, 1-16,000, and 1-32,000 of the starting material. The data from the counts are given in Table I. The particles are shadow-cast with chromium at an angle of about 15°

At an electron magnification of 4,500 x, we can then calculate as follows:

$$\frac{4}{3}\pi r^3 \rho (1-0.392) = \text{g dry wt per particle.}$$

$$\frac{0.00000185 \text{ grams/ml}}{\frac{4}{3}\pi r^3 \rho (1-0.392)} = \text{particles per ml in the 1/1,000 dilution}$$

Equation 1.

where

r = hydrated virus particle radius (58.5×10^{-7} cm)

$1.100 = \rho$ hydrated virus particle density.

1.85×10^{-6} = grams virus per ml in 1/1,000 dilution.

Because of the sector shape of the cell, the number of particles sedimented on unit area of its bottom is not simply the product of the area by the height but 7.1% less, an amount by which the trapezoidal sector area ments virus preparations for electron micrography were made in the usual manner. The suspension of formolized virus was diluted one to ten and prepared for the electron microscope by drying a small amount on the collodion film. Such preparations yielded approximately 25 to 100 virus particles per cm^2 in pictures taken at a magnification of 4,500 x. This was about the concentration of virus ordinarily employed for electron microscope study of the purified virus.

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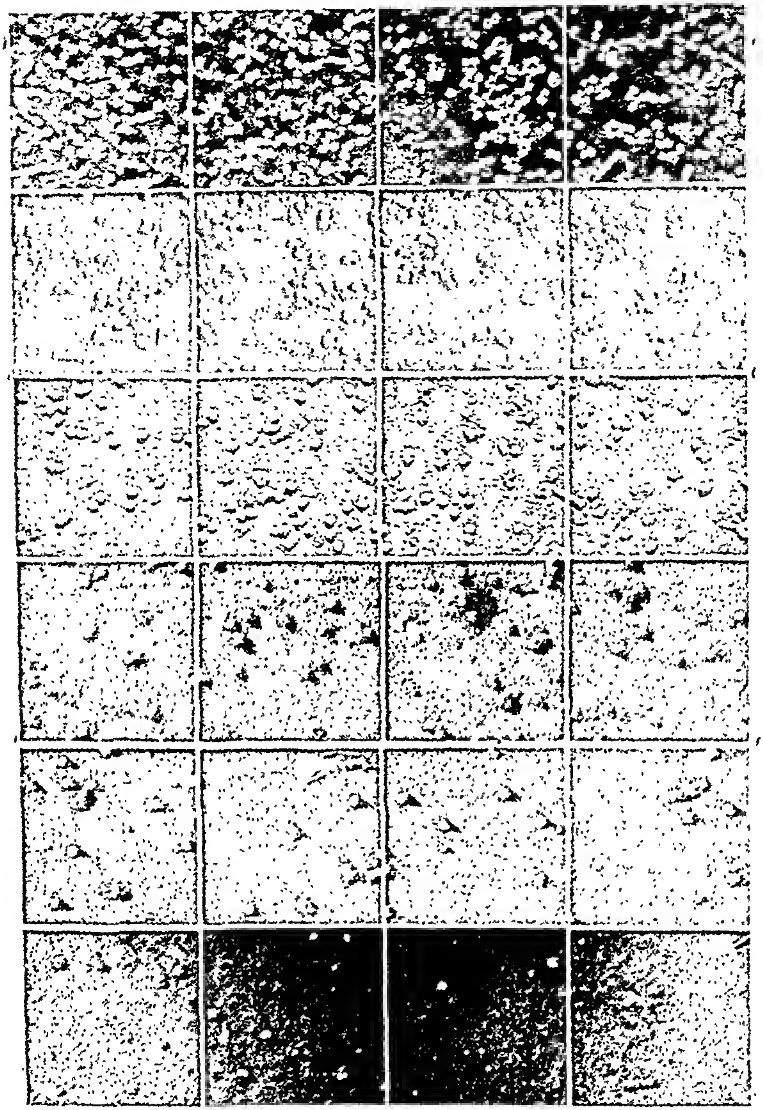

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TABLE I.

Dilution	Total particles counted		Particles observed per cm²		Particles expected Equation 2
	Run I	Run II	Run I	Run II	
1/ 1,000	5,420	—	121 ± 10*	—	151
1/ 2,000	3,418	—	61 ± 7	—	76
1/ 4,000	2,257	1,727	31 ± 6	34 ± 8	38
1/ 8,000	867	863	19 ± 5	16 ± 4	20
1/16,000	415	261	7 ± 2	7 ± 3	10
1/32,000	361	—	4 ± 2	—	5

* Variations are expressed as mean standard deviation. Standard deviation of the mean is of course much less and dependent on the number counted.

is less than that of a rectangle of the same height. The number of images per unit area of a micrograph at 4,500 x is then given by:

$$\frac{3 \times 1.85 \times 10^{-6} \times 0.929}{4\, \pi\, r^3\, 1.10\, (1 - 0.392)\, (4{,}500)^2} = 151$$

Equation 2.

The numbers expected for dilutions higher than 1/1,000 are correspondingly less. Clearly these calculated numbers are strongly dependent on accurate knowledge of particle size and electron magnification, for these enter the equation in third and second power respectively. The influence of the other variables is correspondingly less, and it can be judged from Equation 2.

Discussion. From the results shown in Fig. 2 and Table I, it is clear that this method makes possible the counting of the particles of swine influenza virus, yielding numbers closely proportional to the dilution factor of the starting material. Furthermore, agreement is quite good between these numbers and those predicted from calculations involving not only the physical characteristics of the virus particles but also the purity of the starting material. The observed numbers are lower by about 19% than the predicted ones as shown in Table I. Although error in several places may account for this, one source of error is immediately suspected. Not all of the sedimented virus particles might actually remain on the membrane through washing to appear in the final pictures.

The distribution of particles over the collodion membrane as seen in the electron microscope is exceedingly uniform and the pictures obtained in the process come from dilutions of virus 100 to 3,200 greater than those considered optimum for microscopy using standard technics. The advantages of such a method need not be discussed at length. If the results are proved by subsequent work to be strictly quantitative, the possible applications of the procedure are many, not the least of which should be to the study of viruses in preparations of such low virus content that too little of the agent would be present for purification and concentration, and in which the concentration is so low that the usual technics of preparation for microscopy would produce too few virus particles per field to be recognized.

For study of aggregation in suspension, this method should be useful not only for viruses but also for any other suspended material, such as bacteria in liquid media. For such large bodies as bacteria, any horizontal centrifuge carrying a suitable cell could be used for sedimentation of the particles directly on a microscope slide where, after removal from the centrifuge, they could be studied with a light microscope. It would be necessary to calculate the number of pairs, triplets, etc. to be expected from chance sedimentation of two or more particles on the same area of the slide; but when this number is subtracted, the excess would be characteristic of the liquid suspension. The author is not aware of any other satisfactory method of studying this problem.

Summary. A method has been devised for counting virus particles in a suspension by means of the electron microscope. Studies on formolized purified swine influenza virus showed that the particles could be sedimented with a uniform distribution on a collodion membrane in the ultracentrifuge as revealed by electron micrographs of the particles on the

membrane. The findings showed a close correlation with the dilution factor and with the number of particles calculated from chemical and physical data obtained with the virus.

16823

Electronmicroscopy of Cells from Tissue Cultures Infected with Vaccine Virus.

J. WIRTH AND P. ATHANASIU. (Introduced by J. E. Smadel.)
From the Virus Division, Pasteur Institute, Paris.

One of the obvious needs in electronmicroscopy is a satisfactory method for good visualization of intact cells to provide information on their internal structure and the presence of virus particles in infected preparations. Progress in this field has been made in the use of specially prepared histological sections[1] and in the studies of individual avian cells cultured on plastic membranes.[2,3] The present report describes recent observations on the intracellular growth of vaccine virus observed by electronmicroscopy with a modification of the plastic membrane technic.

Materials and methods. The technic for cultivation of mammalian epithelial cells on plastic membranes and their preparation for visualization by the electron microscope has been described in detail elsewhere.[3-5] In brief, this is as follows: .02 cc of a 0.6% solution of "Formvar" (Shawinigan Falls Products Co., Shawinigan Falls, Quebec, Canada) in ethylene chloride is dropped into a petri dish full to overflowing with chilled Ringer's solution. A cover slip held by forceps is inserted under the membrane covering the fluid in the petri dish and the cover slip is raised through the membrane; this provides a smooth non-adherent plastic coating with a distinct growth-differentiating effect; epithelial growth is enhanced and fibroblasts are inhibited.[2,3] Six such cover slips with the plastic membrane facing upward are placed on a specially prepared glass slide. Small tissue explants are placed on each of the 6 plastic membranes. Special nutrient fluid (plasma-extract supernate, embryonic extract and Tyrode) is added to each culture and maintained in place by a small glass ring sealed with vaseline. The 6 cover slips with their membranes and cultures are covered with a petri dish and infected at the start or after growth has taken place. The nutrient medium is changed at 3-4 day intervals if indicated. Strict asepsis is observed throughout the tissue culture procedure. At an appropriate time, usually 3-5 days for vaccinia, the cover slips with the plastic membrane to which are now attached the growing tissue cells are lifted from the culture chamber. A cover slip with the plastic surface facing up is placed in a petri dish containing Ringer's solution; under these conditions the membrane detaches itself completely from the cover slip and floats on the surface. The floating membrane in the petri dish is examined under low power with an ordinary microscope and the desired fields are selected. These are fixed in the center of the metallic mount by a modification[4] of the usual technic employed in electronmicroscopy.

In the present work, minute explants (weighing about 0.1 mg) prepared from the renal cortex of adult rabbits were used. Cultures were inoculated with a washed suspension of neurovaccinia elementary bodies. Elec-

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[1] Richards, A. G., Jr., Anderson, T. F., and Hance, R. T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 148.

[2] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

[3] Wirth, J., and Barski, G., *Ann. Inst. Pasteur*, 1947, **73**, 987.

[4] Wirth, J., *C. R. Acad. Sci.*, 1947, **225**, 899.

[5] Wirth, J., in: Levaditi, C., and Lépine, P., *Traité des Ultravirus des Maladies Humaines*, 2nd edition, Paris, 1948 (Maloine éd.), page 1683.

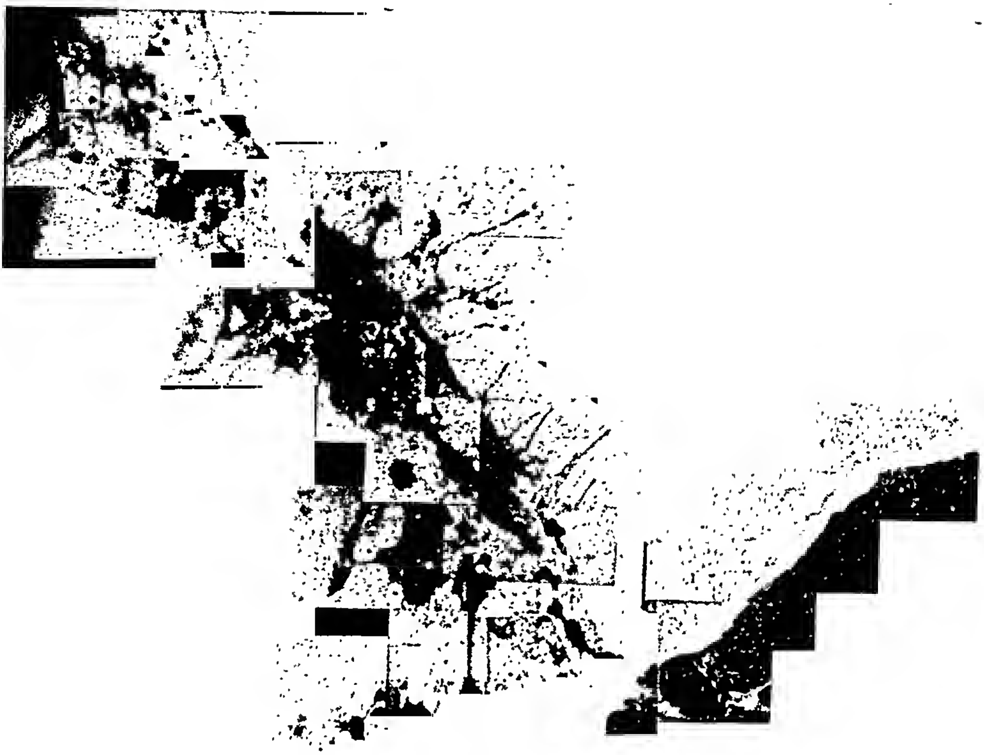

FIG. 1.
Composite electron micrograph of a cell infected with vaccinia. Elementary body-like structures are seen in upper left portion of picture.

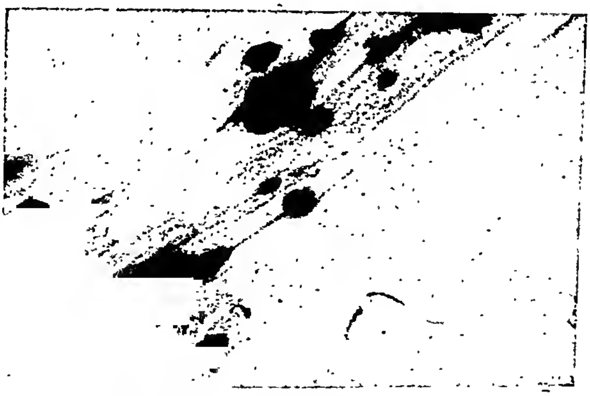

FIG. 2.
Electron micrograph representing a portion of the cell containing numerous elementary body-like structures.

tron micrographs were taken with the C.S.F. electrostatic electron microscope.

Results. Cultures of explants from kidneys of adult rabbits grown by the method described provide essentially pure growth of epithelial cells in a single layer. The technic presents several advantages over the technics previously employed: (1) It provides a relatively simple means for maintaining true cultures a number of days or weeks[6] without transfer; (2) the method does not require the use of a plasma coagulum (fibrin being eliminated); and (3) a monolayer of living cells, almost entirely epithelial, can be obtained for special studies.

A topographical survey of a single cell infected with vaccinia is illustrated in Fig. 1. The individual electron micrographs which make up the composite picture were taken at a magnification of 21,000; after assembly the composite picture was reduced somewhat. The opaque spherical structures occurring in the cytoplasm at the upper left corner are interpreted as elementary bodies of vaccinia seen individually and in clusters. Fig. 2 reproduces one of the micrographs containing the elementary body-like structures which make up Fig. 1. This electron micrograph was also taken at a magnification of 21,000 but has not been reduced. An idea of the reduction in size of the composite picture is obtained by comparing Fig. 2 with the individual components of Fig. 1. Spherical opaque structures such as those observed in Fig. 1 and 2 are regarded as elementary bodies since they have not been observed in similar preparations of uninfected rabbit kidney tissue culture cells. The present method adds another approach to the study of the intracellular growth of vaccine virus which has in the past been fruitfully investigated by microscopic technics employing visible light.[7,8]

Summary. A technic has been devised which provides true cultures of mammalian cells for electronmicroscopy. The usefulness of this procedure in the study of viruses is indicated.

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[6] Barski, G., *Ann. Inst. Pasteur*, 1948, **74**, 312.

[7] Nauck, E. G., and Robinow, C., *Zentralbl. f. Bakt.* (Abt. 1), 1936, **135**, 437.

[8] Bland, J. O. W., and Robinow, C. F., *J. Path. and Bact.*, 1939, **48**, 381.

16824

Adrenalectomy and Pituitary Adrenocorticotrophic Hormone Content.*†

GEORGE SAYERS AND CHI-PING CHENG.‡

From the Department of Pharmacology, University of Utah School of Medicine, Salt Lake City, Utah.

Alterations in the weight and chemical composition of the adrenal gland have been used as quantitative indices for measurement of the rate of discharge of adrenocorticotrophic hormone (ACTH) from the adenohypophysis following application of stress.[1] These indices have been called "target gland indices". It is now possible to complement our knowledge of the pituitary-adrenal system gained from such indices with data on adenohypophyseal content of ACTH. The present report is one of several studies concerned with factors regulating the synthesis and discharge of pituitary ACTH, and deals especially with the changes following adrenalectomy in the rat.

Methods. Male rats from the Sprague-Dawley farm were used. Twenty rats, 230-275 g in body weight, were employed in two experiments conducted at different times. In Experiment I, 6 rats were bilaterally adrenalectomized and 4 rats served as controls; in Experiment II, 4 rats were bilaterally adrenalectomized, 3 rats were subjected to sham adrenalectomy and 3 rats served as controls.

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* Supported by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

† With the statistical assistance of Marion A. Sayers.

‡ Fellow of the American Bureau for Medical Aid to China.

[1] Sayers, G., and Sayers, M. A., *Recent Progress in Hormone Research*, 1948, **2**, 81.

TABLE I.
Pituitary Adrenocorticotrophic Hormone Potency after Adrenalectomy.

Expt.	Donors	Dose of pituitary, μg per 100 g of recipient rats	Reduction of adrenal ascorbic acid content of recipient rat mg per 100 g adrenal tissue
I	Control (4)*	40	146,† 173, 144
		10	84, 94, 59
	Adrenex (6)	40	76, 12, 28
		10	14, 0, 17
II	Control (3)	40	180, 173, 118, 103
		10	68, 26, 18
	Sham (3)	40	150, 117, 112, 125
		10	95, 63, 18, 15
	Adrenex (4)	160	148, 107, 141, 126
		40	64, 25, 54, 81
III	Control (13)	40	147, 189, 147
		20	157, 120, 140
		10	111, 116, 63
		5	97, 26, 26
	Scald (6)	40	158, 133, 151, 147, 174
		20	88, 107
		10	69, 70, 73, 46

* No. of rats in group.
† Each value in right-hand column represents result in one recipient rat.

Twenty-four hours after removal of the adrenals, all rats in the various groups were anesthetized with sodium pentobarbital and the pituitaries removed. During the 24-hour period the animals were fasted but had free access to tap water. A third experiment (III) was conducted in order to compare the changes produced by adrenalectomy with those produced by a severe stress. Nineteen rats weighing 270 to 350 g were fasted 24 hours; 6 were then scalded and the remaining 13 served as controls. All were sacrificed after an additional 24-hour period of fasting. For scalding the rats were immersed up to the neck for 7 seconds in an aqueous detergent ("Duponal") solution at 71 ± 1°C.

The anterior lobes were carefully freed of posterior pituitary tissue, frozen and lyophilized. The pituitaries of each group of rats were pooled, and ground to a homogeneous powder. Aliquots of this powder weighing from about one-half to two mg were extracted successively with 3 portions of a solution of 0.9% NaCl made alkaline to 0.01 N with NaOH. The total volume of extraction fluid was adjusted according to the potency of the tissue and varied from 0.5 ml per 40 μg to 0.5 ml per 160 μg of dry tissue. Appropriate dilutions were prepared for the various dose levels. The extracts were injected into hypophysectomized recipient rats (0.5 ml per 100 g of body weight) and the degree of reduction of adrenal ascorbic acid was measured by the difference between the concentration of ascorbic acid in the left (control) adrenal removed immediately before administration of the pituitary extract and that of the right adrenal removed an hour after such administration.[2]

Results. The assay data from the three experiments are presented in Table I. The ability of extracts of pituitaries to deplete the adrenal ascorbic acid of hypophysectomized recipient rats is a measure of their content of ACTH. It is apparent that the content of ACTH in the adenohypophyses of adrenalectomized rats is markedly reduced. In contrast, the scalded animals exhibit only a slight reduction in ACTH content of their pituitaries.

The results of the 3 experiments have been combined for statistical analysis in Table II. Since 10 μg of pituitary tissue from adrenalectomized animals (Expt. I) failed to give a significant response, the results obtained with this dose have not been included in the analy-

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[2] Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, **42**, 379.

TABLE II.
Relative Content of ACTH in Rat Adenohypophyses.

Groups compared	No. recipient rats	b	s	λ	M	Antilog of M	S_M	Antilog $(M-S_M)$-antilog $(M+S_M)$
Sham (3)*	8	130	29.7	0.228	$\bar{1}.861$	0.73	±0.097	0.58-0.91
Control A (7)	13	149	30.0	0.201				
Adrenex (10)	11	136	24.4	0.180	$\bar{1}.306$	0.20	±0.083	0.17-0.25
Control A (7)	13	149	30.0	0.201				
24-hour scald (6)	11	147	13.9	0.095	$\bar{1}.814$	0.65	±0.069	0.56-0.76
Control B (13)	12	125	27.3	0.218				

b = slope of the log dose response curve; s = standard deviation of all of the individual responses about this curve (a straight line fitted by the method of least squares); λ = s/b and is a measure of the accuracy of the assay method; M = logarithm of the ratio of the potencies; S_M = standard error of M; antilog $(M-S_M)$-antilog $(M+S_M)$ is the range of estimate for one std. error.

* No. rats in donor groups in parentheses.

sis. The 7 control rats of Expt. I and II were combined as "Control A" in Table II. The 13 control rats of the scald experiment (III) serve as "Control B" in Table II. The analyses depicted in Table II include 3 assay pairs, as follows: sham-operated rats compared with Control A; adrenalectomized rats compared with Control A; scalded rats compared with Control B. No significant difference exists between the slopes of the log dose-response curves (b-values) of any two groups compared, a fact which indicates that the observed alterations in biological activity are quantitative and not qualitative. Antilog M, the ratio of the adrenocorticotrophic hormone potencies for each assay pair, is an expression of the relative content of ACTH in the pituitaries of the experimental as compared with the control groups. A slight reduction (27%) of the ACTH content of the pituitary took place in the sham-operated animals. The effects of the scald, acting as a continuous stress over a 24-hour period, reduced the content of ACTH in the adenohypophysis by only 35%. In contrast, adrenalectomy reduced the ACTH content by 80%. The differences in ACTH content cannot be accounted for by differences in the weights of the pituitaries. The mean dry weights of the pituitaries (expressed as mg per rat) were as follows: control A, 0.88; sham-operated, 0.58; adrenalectomized, 0.77; control B, 1.21; scalded, 1.35.

Preliminary histological studies employing the staining technic of Koneff[3] have revealed no striking cytological changes in the anterior pituitary of adrenalectomized rats associated with the marked reduction in ACTH content.

Discussion. In an attempt to elucidate the factors responsible for a reduction in the ACTH content of the pituitary following stress we have formulated the problem in its simplest terms, by assuming that two processes, synthesis (S) and discharge (D) of ACTH, can each separately undergo no change in rate ($_n$), an increase in rate (↑) or a decrease in rate (↓). These 6 variables give rise, theoretically to 9 combinations, represented symbolically as S_nD_n, $S_nD\uparrow$, $S_nD\downarrow$, $S\uparrow D_n$, $S\uparrow D\uparrow$, $S\uparrow D\downarrow$, $S\downarrow D_n$, $S\downarrow D\uparrow$ and $S\downarrow D\downarrow$. Four of these combinations S_nD_n, $S_nD\downarrow$, $S\uparrow D_n$ and $S\uparrow D\downarrow$, are eliminated at once because the problem at hand concerns only a reduction in content of ACTH.

It is possible to rule out 2 more combinations, $S\downarrow D_n$ and $S\downarrow D\downarrow$, on the basis of the fact that they cannot account for the increased rate of discharge of ACTH always associated with stress; studies in this laboratory[1] have shown that a discharge of ACTH follows the subjection of an animal to any of a variety of stresses, such as sham adrenalectomy or scald. Of the remaining 3 combinations, $S_nD\uparrow$, $S\uparrow D\uparrow$ and $S\downarrow D\uparrow$, one does

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[3] Koneff, A. A., *Stain Technol.*, 1938, **13**, 49.

TABLE I.
Pituitary Adrenocorticotrophic Hormone Potency after Adrenalectomy.

Expt.	Donors	Dose of pituitary, μg per 100 g of recipient rats	Reduction of adrenal ascorbic acid content of recipient rat mg per 100 g adrenal tissue
I	Control (4)*	40	146,† 173, 144
		10	84, 94, 59
	Adrenex (6)	40	76, 12, 28
		10	14, 0, 17
II	Control (3)	40	180, 173, 118, 103
		10	68, 26, 18
	Sham (3)	40	150, 117, 112, 125
		10	95, 63, 18, 15
	Adrenex (4)	160	148, 107, 141, 126
		40	64, 25, 54, 81
III	Control (13)	40	147, 189, 147
		20	157, 120, 140
		10	111, 116, 63
		5	97, 26, 26
	Scald (6)	40	158, 133, 151, 147, 174
		20	88, 107
		10	69, 70, 73, 46

* No. of rats in group.
† Each value in right-hand column represents result in one recipient rat.

Twenty-four hours after removal of the adrenals, all rats in the various groups were anesthetized with sodium pentobarbital and the pituitaries removed. During the 24-hour period the animals were fasted but had free access to tap water. A third experiment (III) was conducted in order to compare the changes produced by adrenalectomy with those produced by a severe stress. Nineteen rats weighing 270 to 350 g were fasted 24 hours; 6 were then scalded and the remaining 13 served as controls. All were sacrificed after an additional 24-hour period of fasting. For scalding the rats were immersed up to the neck for 7 seconds in an aqueous detergent ("Duponal") solution at 71 ± 1°C.

The anterior lobes were carefully freed of posterior pituitary tissue, frozen and lyophilized. The pituitaries of each group of rats were pooled, and ground to a homogeneous powder. Aliquots of this powder weighing from about one-half to two mg were extracted successively with 3 portions of a solution of 0.9% NaCl made alkaline to 0.01 N with NaOH. The total volume of extraction fluid was adjusted according to the potency of the tissue and varied from 0.5 ml per 40 μg to 0.5 ml per 160 μg of dry tissue. Appropriate dilutions were prepared for the various dose levels. The extracts were injected into hypophysectomized recipient rats (0.5 ml per 100 g of body weight) and the degree of reduction of adrenal ascorbic acid was measured by the difference between the concentration of ascorbic acid in the left (control) adrenal removed immediately before administration of the pituitary extract and that of the right adrenal removed an hour after such administration.[2]

Results. The assay data from the three experiments are presented in Table I. The ability of extracts of pituitaries to deplete the adrenal ascorbic acid of hypophysectomized recipient rats is a measure of their content of ACTH. It is apparent that the content of ACTH in the adenohypophyses of adrenalectomized rats is markedly reduced. In contrast, the scalded animals exhibit only a slight reduction in ACTH content of their pituitaries.

The results of the 3 experiments have been combined for statistical analysis in Table II. Since 10 μg of pituitary tissue from adrenalectomized animals (Expt. I) failed to give a significant response, the results obtained with this dose have not been included in the analy-

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[2] Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, **42**, 379.

16825

Effect of Injecting Crystalline Tetanal Toxin and Tetanal Antitoxin Into Mice.

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From the Department of Pathology of Northwestern University, Chicago, Ill., and the Institute of Pathology of Western Reserve University, Cleveland, Ohio.

Pillemer and Wartman[1] have shown that the administration of crystalline tetanal toxin in single doses ranging from 0.25 to 500,000 M.L.D. produced no pathological lesions in the muscles or central nervous system of white Swiss mice. Similar results were obtained upon the injection of numerous sublethal doses of toxin. Experiments were subsequently undertaken to determine whether the administration of tetanal antitoxin would produce any pathological lesions in mice that had received various amounts of crystalline toxin. This seemed important because many of the patients with tetanus on whom autopsy was performed have received antitoxin.

Experiments. White Swiss inbred mice, which weighed between 15 and 20 g, were divided into 8 groups. Mice in Groups I to IV were given 1 M.L.D. of crystalline tetanal toxin followed by 25 units of tetanal antitoxin. In Group I the antitoxin was administered one day after the toxin; in Group II 2 days afterwards; in Group III 3 days afterwards; and in Group IV 4 days afterwards. Mice in Group V were given 0.5 M.L.D. of toxin followed 5 days later by 25 units of antitoxin. In Group VI the procedure was reversed and the mice were given first 25 units of antitoxin and then 1 M.L.D. of toxin every day for 6 successive days, receiving in all 6 M.L.D. of toxin. Animals in Group VII were given 25 units of antitoxin, and those in Group VIII 2500 units of antitoxin. None of the mice in Groups VII and VIII received tetanal toxin.

Crystalline tetanal toxin[2,3] and a highly purified antitoxin (Lederle) were used in all experiments. The toxin was dissolved in 0.3 M glycine and injected into the gluteal muscles at the base of the tail on the right side. The antitoxin was diluted in 0.9% saline and injected intraperitoneally. The mice were amply fed and allowed as much water as they would drink.

The mice were killed at the desired intervals with ether, except those in Groups III and IV which died. Autopsies were performed immediately after death, and tissues were fixed for 24 hours in 10% formalin and Zenker's fluid. Sections were stained with hemalum and aqueous eosin and, in addition, the brain, spinal cord, and peripheral nerves were stained specifically for Nissl substance and myelin. Sections were made of the following organs: the anterior, middle, and posterior thirds of the brain; the lumbar, thoracic, and cervical portions of the spinal cord; anterior and posterior nerve roots; sciatic nerve; the cerebral and spinal meninges; the skeletal muscles from both the injected and the opposite side of the tail, both gastrocnemius muscles, and the muscles along the vertebral column.

Results. When 25 units of tetanal antitoxin were given to white Swiss mice within 24 hours after the intramuscular injection of 1 M.L.D. of crystalline tetanus toxin, the animals were protected from the development of clinical tetanus. When the antitoxin was not given until later, the signs of classical tetanus developed and the mice died. No detectable pathological changes occurred in animals which survived for as long as 14 days. If only 0.5 M.L.D. of toxin was injected, the administration of 25 units of antitoxin was effective even if delayed for several days. When given as long as 5 days after the toxin, some of the mice developed not seem probable, namely, S↓D↑, because it appears most unlikely that the rate of synthesis of ACTH would be depressed when there is an increased body need for the hormone. Thus only two combinations remain to be considered as possibilities, namely, (1) increased discharge of ACTH associated with no change in the rate of synthesis, and (2) increased discharge associated with an increased rate of synthesis.

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[1] Pillemer, L., and Wartman, W. B., *J. Immunol.*, 1947, **55**, 277.

[2] Pillemer, L., Wittler, R. G., and Grossberg, D. B., *Science*, 1946, **103**, 615.

[3] Pillemer, L., Wittler, R. G., Burrell, J. L., and Grossberg, D. B., *J. Exp. Med.*, 1948, **88**, 205.

It is likely that in mild stress the first possibility obtains; in severe stress, the second possibility. The pituitary stores a relatively large quantity of ACTH; there is an amount of ACTH in one rat pituitary sufficient to produce a marked reduction (50% of maximal) in the ascorbic acid content of the adrenals of 100 rats. Mild stress produces a reduction in adrenal ascorbic acid content which is approximately 50% of maximum. Therefore, the quantity of ACTH normally stored in the pituitary is 100-fold greater than the amount required to meet the immediate needs of the organism subjected to mild, short-acting forms of stress; synthesis of new hormone would not be needed in order to cope with the stressful situation. However, in the case of severe, long-acting forms of stress, for example, scald, synthesis must be accelerated so that the store of ACTH can be fairly well maintained despite its rapid and continuous discharge. In Table II, it can be seen that the ACTH content of scalded rats was maintained at 65% of the control level.

In contrast, adrenalectomy is followed by a very marked reduction in the pituitary content of ACTH. Whereas the severe continuous stress of scalding resulted in a reduction of only 35%, adrenalectomy led to a reduction of 80% in hormone content. Unfortunately, direct experimental evidence regarding rate of discharge of ACTH, such as that available in the stress experiments, was not obtainable in the adrenalectomy experiments, because adrenalectomized animals lack the target gland which provides a simple means for measuring rate of ACTH discharge. Therefore, one must consider depression of synthesis as well as increased rate of discharge as a factor responsible for the depletion of stores of ACTH in the pituitary 24 hours after adrenalectomy. However, we are inclined to favor the view that the rate of discharge of ACTH is at a maximum when there is a complete absence of the target gland secretion. This latter possibility is in keeping with other experimental data from this laboratory[4] which have been interpreted to mean that the rate of discharge of ACTH increases as the level of cortical hormones in the body fluids falls. According to this concept, adrenalectomy provides a maximum stimulus for discharge of ACTH since it produces the lowest possible titers of cortical steroids in body fluids. Determination of body fluid titers of ACTH in adrenalectomized animals by direct assay would be of considerable help in assessing the relative roles of synthesis and discharge as factors regulating the ACTH stores of the pituitary.

Summary. Twenty-four hours after adrenalectomy the content of ACTH in the pituitary of the rat is reduced by 80%. Sham adrenalectomy and scalding reduce the content of ACTH by only 27% and 35%, respectively. The significance of these observations for the problem of the regulation of rate of synthesis and discharge of pituitary ACTH is discussed.

The authors are indebted to Dr. Louis S. Goodman and Dr. Walter S. Loewe for their many helpful suggestions and criticisms during the preparation of the manuscript.

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[4] Sayers, G., and Sayers, M. A., *Endocrinology,* 1947, 40, 265.

TABLE I.

Effect of Injections of Pituitary Glands Obtained from Lithosperm and Control Donors on Organs of 21-day-old Mice.

Group	No. mice	Organ weight in mg			Body weight in g	
		Ovaries	Uterus + vagina	Adrenals	21 days old	26 days old
I. Lith. donors	9	5.0 ± 0.3	14.6 ± 1.1	2.4 ± 0.2	6.9 ± 0.5	9.0 ± 0.7
II. Control donors	9	5.8 ± 0.4	26.2 ± 4.0	2.7 ± 0.2	6.9 ± 0.2	9.5 ± 0.3
III. Uninjected controls	10	4.8 ± 0.3	13.5 ± 1.7	1.9 ± 0.2	6.9 ± 0.6	8.6 ± 0.9

not seen in mice receiving pituitary injections from Lithosperm donors (Group I) inasmuch as there is no significant difference between the weights of the uterus plus vagina in these mice and in the uninjected controls (Group III). On the other hand considerable gonadotropic stimulation was evident in the mice receiving pituitary injections from control donors (Group II), the uterus plus vagina weighing 95% more than that of the uninjected controls and 79% more than that of the Lithosperm donor. These differences are significant statistically. Thus the results show that the gonadotropic potency of pituitary glands of adult female mice receiving Lithosperm was decidedly less than that of similar control mice.

No significant differences in the weights of ovaries or adrenal glands or in body weight between the 3 groups were obtained. Had the mice been injected with more pituitary glands, some differences might have been noted, at least in ovarian weight. Increase in the weight of ovaries is also an indicator of gonadotropic stimulation, but a less sensitive one than that of the uterus.[4]

The concentration of the sample of Lithosperm (20%) used in this experiment is a threshold one, producing continuous diestrous in 55 per cent of mice of a sensitive strain (C_3H). A concentration of 30% is required to produce continuous diestrous in all such mice.

Discussion. The finding that Lithosperm decreases the gonadotropic potency of the pituitary gland aids in the understanding of the mechanism of action of this crude drug. The contention that the action is not a direct one on the vagina, ovaries or circulating gonadotropin but involves rather the gonadotropic activity of the pituitary gland is strengthened. Zahl[5] has shown that no histological changes are evident in the pituitary gland after prolonged Lithosperm administration and that normal estrous cycles are reestablished quickly after discontinuance of the drug, even after 8 months of therapy. Thus it appears that Lithosperm inhibits in some way the formation of gonadotropic hormone without producing irreversible or histological damage to the pituitary gland.

Conclusion. The gonadotropic potency of pituitary glands of adult female mice is decreased by the administration of *Lithospermum ruderale* as evidenced by a lack of increase in weight of the uterus plus vagina in 21-day-old mice which have been injected with pituitary glands from treated mice.

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[4] Levin, L., and Tyndale, H. H., *Endocrin.*, 1937, **21**, 619.

[5] Zahl, P. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 405.

tetanus, but the signs were only mild to moderate. In these mice, also, no significant pathological lesions were discovered.

The intraperitoneal injection of either 25 or 2500 units of tetanal antitoxin caused no recognizable clinical or pathological manifestations.

Summary. The administration of 25 units of tetanal antitoxin to white Swiss mice which had previously received 1 M.L.D. of crystalline tetanal toxin, produced no discoverable lesions in the central nervous system, peripheral nerves, or skeletal muscles, irrespective of whether the animals developed clinical tetanus, or of how long they survived. Likewise, the intraperitoneal injection of either 25 or 2500 units of tetanal antitoxin caused no changes which could be detected by ordinary histopathological technics.

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Effect of *Lithospermum ruderale* on the Gonadotropic Potency of the Pituitary Gland.*

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(Introduced by Raymond N. Bieter.)

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The crude plant, *Lithospermum ruderale*, has been shown to cause prolonged or persistent diestrous in mice with previously regular estrous cycles.[1,2] Since the administration of either follicle-stimulating hormone or estrogenic hormone induces estrus in animals under treatment with Lithosperm and since Lithosperm causes a decrease in the weights of pituitary glands the suggestion has been made that the drug acts on the pituitary gland to decrease the formation or secretion of gonadotropic hormone.[1] The present paper deals with the biological assay for gonadotropic potency of pituitary glands from treated and control animals.

Experimental. Female mice, 21 days old, were divided into 3 groups, distributing littermates between the groups as evenly as possible. Group I called *Lithosperm donors*, consisted of 9 mice, each of which was given an injection of two pituitary glands from donor mice which had been on a 20% ground Lithosperm diet for one week or more. In Group II, the control donors, each of 9 mice received an injection of 2 pituitary glands from donor mice on control diet, Purina fox chow. Group III served as uninjected controls, receiving no pituitary injections. Donor mice for Groups I and II were adult females and were sacrificed during diestrus only. To make the pituitary injections a method similar to that of Smith and Engle[3] was used. Donor mice were killed with ether, the skin on back of head calvarium and brain removed and pituitary gland lifted out. Recipient mice were lightly anesthetized with ether and the pituitary glands placed through a small incision into the thigh muscles. Mice were autopsied at 26 days of age, *i.e.* 5 days after injection. The ovaries, uterus plus vagina and adrenal glands were dissected out and weighed in the moist state.

The data obtained are presented in Table I. Evidence of gonadotropic stimulation was

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* This work constitutes part of a study supported by a grant from the U. S. Public Health Service.

[1] Cranston, E. M., *J. Pharm. and Exp. Therap.*, 1945, 83, 130.

[2] Drasher, M. L., and Zahl, P. A., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 66.

[3] Smith, P. E., and Engle, E. T., *Am. J. Anat.*, 1927, 40, 159.

TABLE I. Weight Changes of Thymus and Lymph Nodes in Intact Mice Bearing Sarcoma 180.

Sex	Group	No. animals	Final body wt, g	Thymus wt mg	Thymus wt mg/100 g B.W.	Lymph nodes wt mg	Lymph nodes wt mg/100 g B.W.
Male	Tumor bearing	10	16.1 ± 0.5*	9.8 ± 1.9	61 ± 10.5	54.4 ± 5.4	338 ± 28.9
	Controls	12	18.1 ± 0.5	27.0 ± 2.7	149 ± 12.5	30.5 ± 2.7	169 ± 13.3
Female	Tumor bearing	10	16.0 ± 0.5	16.4 ± 2.5	102 ± 14.0	63.6 ± 0.4	398 ± 19.4
	Controls	12	17.6 ± 0.5	30.9 ± 2.4	176 ± 12.9	34.8 ± 0.4	198 ± 20.3

* Mean ± standard error.

TABLE II. Weight Changes of Thymus and Lymph Nodes in Hypophysectomized Mice Bearing Sarcoma 180.

Sex	Group	No. animals	Final body wt, g	Thymus wt mg	Thymus wt mg/100 g B.W.	Lymph nodes wt mg	Lymph nodes wt mg/100 g B.W.
Female	Tumor bearing	7	14.8 ± 1.2	26.1 ± 1.9	176 ± 12.5	65.9 ± 2.5	445 ± 17.1
	Controls	10	14.4 ± 0.3	36.4 ± 2.2	252 ± 14.3	45.0 ± 3.1	312 ± 22.8

Twelve days following tumor implantation, all animals were sacrificed by cervical fracture following a 16 hour fast, during which water remained available. The thymus and 3 lymph nodes (one axillary node from each side and one mesenteric node) were dissected from each animal and immediately weighed separately; other tissues were removed from these mice for additional studies which will be reported later. The weight of thymus and the combined weights of the three nodes, expressed in mg of fresh tissue and as mg per 100 g of final body weight are given in Table I.

In a second experiment, female mice of the same strain, weighing 12 to 14 g were hypophysectomized. Five days later Sarcoma 180 was implanted in the usual way in one half of the animals, the remainder serving as controls. From the time of pituitary removal the mice were maintained as described above except for the replacement of drinking water by physiological saline solution. The animals were allowed to survive for ten days following implantation and during this time one half of the tumor bearing group had died, while only 2 of the hypophysectomized control group were lost. Both groups were then sacrificed and the tissues dissected and weighed as described above. The completeness of pituitary removal, already indicated by the cessation of growth over the experimental period, was verified by histological examination of the base of the skull. Table II lists the weights of the thymus and the combined weights of the 3 lymph nodes of the hypophysectomized animals.

Discussion. The data shown in Table I and Fig. 1 demonstrate that mice of the CFW strain, bearing Sarcoma 180, exhibit atrophy of the thymus averaging 50% by weight, and an increase in lymph node weight of the order of 100%. No important sex difference was noted. Visual inspection indicated that the weight increase in the selected lymph nodes reflected a generalized change in the entire lymphatic system of the tumor bearing mice, a fact which has since been confirmed.[23] The

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[23] Savard, K., and Tompkins, M., unpublished data.

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Thymic Atrophy and Lymphoid Hyperplasia in Mice Bearing Sarcoma 180.

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The changes in the size or weight of the thymus induced by various endocrine factors have been assumed to parallel those occurring in lymph nodes.[1-4] This is instanced by the concurrent hypertrophy of these tissues following adrenalectomy[5,6] and the concurrent atrophy after thyroidectomy,[7,8] gonadectomy,[7,9] the administration of cortical hormones[10-12] and pituitary adrenocorticotrophin.[13-15] While this evidence strongly supports the contention that the thymus acts as part of the lymphoid tissue, opinions to the contrary are widely held[16-18] and have recently been reviewed.[19]

One of us recently described[20] the *pronounced hyperplasia* of, and the increased nitrogen deposition in lymphoid tissue (as represented by the combined thymus and lymph node weight and nitrogen content) of male mice of the CFW strain bearing implants of Sarcoma 180. Independently, the other of us observed that Sarcoma 180 in female mice of the same strain evoked a *significant atrophy* of the thymus.[21] It thus appeared that under the conditions of these experiments identical factors could produce a *simultaneous thymic atrophy and lymphatic hyperplasia.* The present study demonstrates this fact.

Experimental. Sarcoma 180 was implanted subcutaneously near the axilla[22] as previously described[20,21] into 12 male and 12 female CFW mice (Carworth Farm) of approximately 18-20 g body weight; an equal number of untreated mice of both sexes served as controls. The latter group (6 animals per cage) received an amount of Purina Fox Chow equivalent to that consumed by the corresponding tumor bearing group and all animals were given tap water *ad libitum;* this control of food intake was later found to have no apparent effect on the tissue changes observed.

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[†] Present address: Cancer Research and Cancer Control Unit, Tufts College Medical School and New England Medical Center, 30 Bennet Street, Boston, Mass.

[1] Dougherty, T. F., and White, A., *J. Lab. Clin. Med.*, 1947, **32**, 584.

[2] Houssay, B. A., quoted *J.A.M.A.*, 1942, **118**, 833.

[3] Li, C. H., and Evans, H. M., *Vitamins and Hormones, Advances in Research and Applications*, 1947, **5**, 197, Academic Press, Inc., New York.

[4] Selye, H., *J. Clin. Endocrinology*, 1946, **6**, 117.

[5] Reinhardt, W. O., and Holmes, R. O., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 267.

[6] Houssay, B. A., *Rev. Soc. Argentina Biol.*, 1941, **17**, 26.

[7] Chiodi, H., *Endocrinology*, 1940, **26**, 107.

[8] Reinhardt, W. O., and Wainman, P., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 257.

[9] Chiodi, H., *Rev. Soc. Argent. de Biol.*, 1938, **14**, 74.

[10] Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 443.

[11] Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 174.

[12] Dougherty, T. F., and White, A., *Am. J. Anatomy*, 1945, **77**, 81.

[13] Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 42.

[14] Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

[15] Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 135.

[16] Best, C. H., and Taylor, N. B., Physiological Basis of Medical Practice, Williams and Wilkins Co., Baltimore, 1943.

[17] Caffey, J., The Nelson Loose-Leaf Medicine, Thomas Nelson and Sons, New York, 1946.

[18] Margolis, H. M., *Archiv. Path.*, 1930, **9**, 1015.

[19] Andreasen, E., *Acta Pathol. et Microbiol. Scandinavia*, Suppl. XLIX, Dissertation 1943.

[20] Homburger, F., *Science*, 1948, **107**, 648.

[21] Savard, K., *Science*, 1948, **107**, 381.

[22] Sugiura, K., *Radiology*, 1937, **28**, 162.

TABLE I. Weight Changes of Thymus and Lymph Nodes in Intact Mice Bearing Sarcoma 180.

Sex	Group	No. animals	Final body wt, g	Thymus wt		Lymph nodes wt	
				mg	mg/100 g B.W.	mg	mg/100 g B.W.
Male	Tumor bearing	10	16.1 ± 0.5*	9.8 ± 1.9	61 ± 10.5	54.4 ± 5.4	338 ± 28.9
	Controls	12	18.1 ± 0.5	27.0 ± 2.7	149 ± 12.5	30.5 ± 2.7	169 ± 13.3
Female	Tumor bearing	10	16.0 ± 0.5	16.4 ± 2.5	102 ± 14.0	63.6 ± 0.4	398 ± 19.4
	Controls	12	17.6 ± 0.5	30.9 ± 2.4	176 ± 12.9	34.8 ± 0.4	198 ± 20.3

* *Mean* ± standard error.

TABLE II. Weight Changes of Thymus and Lymph Nodes in Hypophysectomized Mice Bearing Sarcoma 180.

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Female	Tumor bearing	7	14.8 ± 1.2	26.1 ± 1.9	176 ± 12.5	65.9 ± 2.5	445 ± 17.1
	Controls	10	14.4 ± 0.3	36.4 ± 2.2	252 ± 14.3	45.0 ± 3.1	312 ± 22.8

Twelve days following tumor implantation, all animals were sacrificed by cervical fracture following a 16 hour fast, during which water remained available. The thymus and 3 lymph nodes (one axillary node from each side and one mesenteric node) were dissected from each animal and immediately weighed separately; other tissues were removed from these mice for additional studies which will be reported later. The weight of thymus and the combined weights of the three nodes, expressed in mg of fresh tissue and as mg per 100 g of final body weight are given in Table I.

In a second experiment, female mice of the same strain, weighing 12 to 14 g were hypophysectomized. Five days later Sarcoma 180 was implanted in the usual way in one half of the animals, the remainder serving as controls. From the time of pituitary removal the mice were maintained as described above except for the replacement of drinking water by physiological saline solution. The animals were allowed to survive for ten days following implantation and during this time one half of the tumor bearing group had died, while only 2 of the hypophysectomized control group were lost. Both groups were then sacrificed and the tissues dissected and weighed as described above. The completeness of pituitary removal, already indicated by the cessation of growth over the experimental period, was verified by histological examination of the base of the skull. Table II lists the weights of the thymus and the combined weights of the 3 lymph nodes of the hypophysectomized animals.

Discussion. The data shown in Table I and Fig. 1 demonstrate that mice of the CFW strain, bearing Sarcoma 180, exhibit atrophy of the thymus averaging 50% by weight, and an increase in lymph node weight of the order of 100%. No important sex difference was noted. Visual inspection indicated that the weight increase in the selected lymph nodes reflected a generalized change in the entire lymphatic system of the tumor bearing mice, a fact which has since been confirmed.[23] The

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[23] Savard, K., and Tompkins, M., unpublished data.

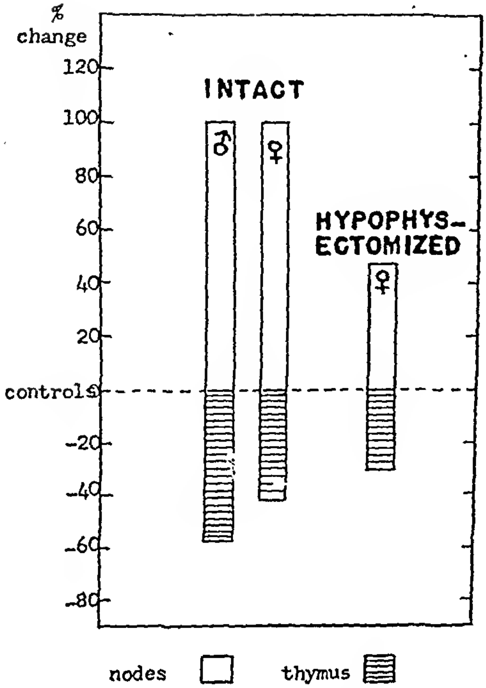

FIG. 1.

Per cent change over control values of thymus and lymph nodes weights expressed as mg per 100 g of body weight. The columns on the left refer to the intact animals; the one on the right refers to the hypophysectomized mice.

possibility of the hyperplasia being due to infection, regional lymphadenopathy or metastases of the tumor has been excluded in an earlier series by histological studies and a true lymphatic hyperplasia established by the clear cut increase in nitrogen content of these nodes.[20] There was thus, under the conditions of the experiment, a distinct dissociation of the response of the thymus and of the lymph nodes to the presence of growing Sarcoma 180.

In view of the fact that the growth of Sarcoma 180 in the CFW strain of mice is accompanied by adrenal changes indicative of an increased stimulation of the latter organ by the anterior pituitary[21] the possibility of a relationship between the endocrine system and the lymphatic and thymic changes was considered. The results of the second experiment shown in Table II indicate however that in the hypophysectomized animal, the growing tumor evoked a thymic atrophy and lymphatic hyperplasia, though to a lesser degree than in the intact animal; the adrenal hypertrophy and lowered ascorbic acid levels induced by the tumor in the intact animal[21] were, on the other hand, not observed in the hypophysectomized mice.[23] It is thus implied that the thymic and lymphatic changes are not mediated through the pituitary-adrenal system.

Summary. Transplanted Sarcoma 180 after 12 days of growth evokes thymic atrophy and lymphatic hyperplasia in mice of the CFW strain. These changes are not prevented by hypophysectomy, thereby excluding pituitary-adrenal mediation.

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16828

Response of Guinea Pigs to Diets Deficient in Choline.

PHILIP HANDLER.

From the Department of Biochemistry, Duke University School of Medicine, Durham, N. C.

The hepatic choline concentration of the choline-deficient rat, when calculated on a fat-free basis, is not lower than that of normal rats.[1,2] This fact has been correlated with the observation that the choline oxidase activity of the fatty livers of choline deficient rats is markedly suppressed[3] and the suggestion advanced that it is the diminished choline

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[2] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 357.

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TABLE I.
Composition of Diets.

	1	2	3	4	5	6	7
Casein	12	12	12	12	10	6	6
Cellulose	20	20	10	20	20	15	10
Salts[18]	4	4	4	5	5	5	5
Liver "L"	3	3	3	5	5	5	4
Lard	10	10	15		20	15	25
Glucose	48	48	52	55			
Sucrose					23	21	15
Cod liver oil	3	3	3	1			
Cystine	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Cholesterol	0.3	0.3	0.3	0.3	0.5	0.5	0.5
Inositol	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Glycocyamine	0.1	0.3	0.3	0.3	0.5	0.5	0.3
Arginine				1.0	1.0	1.0	0.5
Glycine				0.3	0.3	0.3	0.3
Peanut Meal						30	30
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oxidase activity which permits the existence of a normal hepatic choline concentration (as phospholipid) despite the dietary choline deficiency.[1,3] Choline deficiency and its manifestations have been most frequently studied in the rat[4] but have also been observed in the dog,[5] mouse,[6] and chick[7] as well as implicated in the etiology of hepatic cirrhosis in man. The livers of these species have all been shown to possess marked choline oxidase activity.[8] Although fatty livers have frequently been observed in the guinea pig, under various conditions there has been, as yet, no demonstration that these have been, in any case, the result of dietary choline deficiency. Since the liver of this species is unique among common laboratory animals in that it possesses little or no choline oxidase activity,[8] it was of considerable interest to determine the susceptibility of this species to dietary choline deficiency. The present paper describes a number of attempts to elicit some manifestation of choline deficiency in young guinea pigs under conditions which were quite effective for the rat.

Experimental. In preliminary studies various procedures for the feeding of synthetic rations to young guinea pigs were investigated. In some instances the newborn pigs were offered the experimental diets while still suckling and were continued on them after separation from the sow. A few attempts were made to offer such rations to one-week-old pigs weaned at that time. However, the data to be presented were all obtained in the following manner. Weanling guinea pigs were immediately offered a diet consisting of equal parts of the synthetic ration and minced carrots. After 4 days the amount of carrots was reduced by half and 4 days later the carrots were removed entirely. A group of rats, as controls, was also offered each of the experimental rations in similar fashion. All animals were housed in individual cages with food and water available *ad libitum*. Three weeks after deletion of the carrots from the diet the animals were sacrificed by decapitation and their livers taken for lipid analysis.[9]

Using this feeding technic relatively few young pigs refused the experimental rations. All animals which failed to eat adequately or which lost weight unduly and died before termination of the feeding trial were discounted in evaluating the results of these experiments. This was considered valid since approximately equal numbers of pigs died on both choline-deficient and choline-supplemented rations. It is of interest that, in many instances, on each of the experimental rations employed, pigs died suddenly after a

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[4] Best, C. H., and Lucas, C. C., *Vitamins and Hormones*, 1943, **1**, 1.

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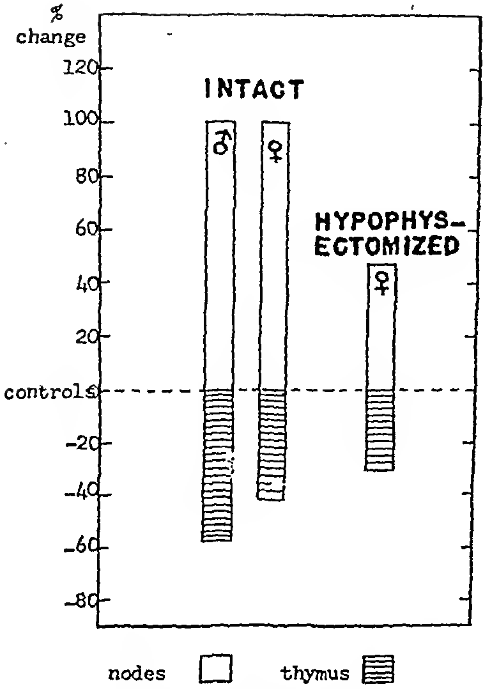
[6] Handler, P., unpublished data.

[7] Jukes, T. H., *J. Nutrition*, 1940, **20**, 445.

[8] Bernheim, F., and Bernheim, M. L. C., *Am. J. Physiol.*, 1938, **121**, 55.

[9] Handler, P., *J. Biol. Chem.*, 1948, **173**, 295.



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Per cent change over control values of thymus and lymph nodes weights expressed as mg per 100 g of body weight. The columns on the left refer to the intact animals; the one on the right refers to the hypophysectomized mice.

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[9] Handler, P., *J. Biol. Chem.*, 1948, **173**, 295.

TABLE II.
Liver Lipids of Guinea Pigs and Rats on Choline-Deficient Diets.

Group	Species	No. animals	Diet	Initial wt, g	Wt change, g	Food intake, g/day	Liver lipids, % wet wt
1	GP	6	1	188	— 7	9.3	4.8
2	"	5	1 + choline	181	— 6	10.0	4.6
101	Rat	6	1	82	29	8.3	11.6
102	"	6	1 + "	76	31	8.1	5.3
3	GP	5	2	189	2	9.2	4.4
4	"	6	2 + "	196	— 3	9.6	4.1
103	Rat	5	2	68	26	7.7	13.8
104	"	5	2 + "	73	23	7.9	6.1
5	GP	7	3	161	—11	9.1	4.3
6	"	6	3 + "	173	— 6	9.9	4.4
105	Rat	6	3	84	16	7.2	15.6
106	"	6	3 + "	76	24	7.9	5.9
7	GP	5	4	143	9	8.7	4.9
8	"	8	4 + "	148	4	8.4	5.2
107	Rat	6	4	61	29	8.0	10.8
108	"	6	4 + "	60	27	8.0	4.7
9	GP	6	5	148	—13	6.9	5.6
10	"	6	5 + "	156	—17	7.1	5.3
109	Rat	5	5	69	17	6.3	21.7
110	"	5	5 + "	64	23	6.1	6.3
11	GP	7	6	173	41	12.7	7.4
12	"	6	6 + "	184	37	11.9	7.7
111	Rat	6	6	67	16	6.7	24.2
112	"	6	6 + "	72	23	6.6	5.8
13	GP	6	7	177	47	11.8	8.3
14	"	6	7 + "	189	41	11.4	6.9
15	"	5	7 + methionine	180	40	11.5	8.2
113	Rat	5	7	74	14	7.0	26.7
114	"	5	7 + choline	61	19	6.9	6.8

variable period of reasonable weight gain or maintenance and apparently adequate food consumption. Similar deaths have not been observed in animals living on the stock diets commonly employed for this species.

The various diets employed are summarized in Table I. Each diet was offered to a group of control rats as well as to guinea pigs. To each kilo of each diet was added thiamine 10 mg, riboflavin 12 mg, pyridoxine 10 mg, *p*-aminobenzoic acid 25 mg, calcium pantothenate 50 mg, niacin 50 mg, ascorbic acid 200 mg, α-tocopherol 100 mg, and 2-methyl-1, 4-naphthoquinone 10 mg. All animals received 50 γ biotin and 50 γ folic acid 3 times weekly by pipette. All animals not receiving cod liver oil in the diet were given 2 drops of percomorph oil 3 times weekly. All animals on diets 3 - 7 were given 1 drop of cream 3 times weekly by pipette. Not indicated in the tables is the fact that each of these diets was also fed without inositol. However, in no case did the absence of this substance appear to affect the results. Choline, when present was incorporated as 0.6% of the diet as was DL-methionine. The results are summarized in Table II.

The inclusion of liver fraction "L" in all diets was based on the findings of Woolley and Sprince[10] and has been found to contain but 0.87 mg of choline per gram. Arginine and glycine were added to diets 4 - 7 as these have been found to improve the growth of guinea pigs on low protein rations.[11] Glycocyamine was added to diets 2 - 7 since, like nicotinamide, it forces a demand for methyl groups and increases the choline and/or methionine requirement of the rat.[12] "Cerophyl" was included in diet 7 as a source of "grass juice factor". The rations containing alcohol-extracted peanut meal were patterned after those used by Engel and Salmon[13] and proved to be the most successful diets in this study. It will be seen that most of the guinea pigs on diets 1 - 5 grew very poorly or not at all and a considerable number actually lost weight during the experimental period. It has been noted that under such conditions little or no fat accumulates in rat livers despite choline deficiency[2,14,15] and it is now well recognized that the optimal conditions for eliciting the hepatic and renal manifestations of choline deficiency are those which permit the maximum growth rate compatible with the relatively low protein diets necessary in such studies.[16] The peanut meal rations 6 and 7 much more satisfactorily met these requirements as seen in Table II.

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[10] Woolley, D. W., and Sprince, H., *J. Biol. Chem.*, 1944, **153**, 687.

[11] Woolley, D. W. personal communication.

It is apparent that while diets 1 - 5 were sufficiently lacking in choline and methionine to produce fatty livers in rats, they did not result in the accumulation of fat in guinea pig livers. Diets 6 and 7, which were the most effective of this series in producing fatty livers in rats, also resulted in a slight elevation of the fat content of the guinea pig livers. However, this was not diminished by the inclusion of choline or methionine in these diets and, consequently, this effect cannot be ascribed to choline deficiency.

The data presented herein should not be interpreted as indicating that the metabolism of phospholipids in the guinea pig is radically different from events in the rat. The possibility exists that the conditions of these experiments permitted sufficient intra-intestinal synthesis of labile methyl compounds to meet the animals' requirements. However, the failure of glycocyamine, like that of nicotinamide,[17] to increase the demand for a supply of methyl groups sufficiently to result in fatty liver formation is surprising. The present findings are compatible with the lack of hepatic choline oxidase activity in this species and suggest that the lack of this enzyme results in a choline turnover so slow as to permit a nutritionally adequate supply of choline from such choline and methionine as was available in the diets of this study. While it may yet be found that the position of choline in the nutrition of the guinea pig is much like that in the rat, the daily requirement of the guinea pig for choline (or its equivalent in methyl groups) is certainly of a lower order of magnitude than that of the rat.

Summary. Seven different choline deficient diets were fed to young rats and guinea pigs. While each of these diets resulted in fatty liver formation in the rats, in no instance was there observed an appreciable accumulation of fat in the guinea pig livers. This fact has been correlated with the lack of hepatic choline oxidase activity in the guinea pig as compared to all species which have, to date, been found susceptible to dietary choline deficiency.

The author's thanks are due to the Duke University Research Council and to The Nutrition Foundation for their support of this work; to Merck and Co., Rahway, N. J., for the crystalline vitamins employed; and to the Wilson Laboratories, Chicago, Ill., for a generous supply of liver fraction L.

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[12] Stetten, DeW., and Grail, G. F., *J. Biol. Chem.*, 1942, **144**, 175.

[13] Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

[14] Handler, P., *J. Biol. Chem.*, 1943, **149**, 291.

[15] Handler, P., *J. Biol. Chem.*, 1946, **162**, 77.

[16] Griffith, W. H., *Biol. Symposia*, 1941, **5**, 193.

[17] Handler, P., *J. Biol. Chem.*, 1944, **154**, 503.

16829

Glutamic Acid and Vomiting in Dogs: Its Administration into the Portal System and Extremity Veins.*

ANDREW G. LASICHAK AND STANLEY LEVEY. (Introduced by A. H. Smith.)

From Wayne County General Hospital, Eloise, Mich., and the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit, Mich.

The intravenous administration of solutions containing glutamic acid has been shown to produce vomiting both in dogs[1] and in man.[2] Price, Waelsch and Putnam,[3] on the other hand, have given as much as 20 g of glutamic acid orally to epileptic patients per day in divided doses and did not record any incidence of vomiting. The increased tolerance to orally administered glutamic acid may be due either to slow absorption from the digestive tract, or to the fact that products which are absorbed from the gastro-intestinal tract by way of the blood are first conveyed to the liver. In this organ many reactions occur which could destroy free glutamic acid, such as deamination, transamination, or peptide formation. In order to study the detoxifying action of the liver on glutamic acid two methods of administration were used whereby the infusions would enter the portal system before reaching the general circulation. This was accomplished by infusing the solutions containing glutamic acid either directly into the spleen or the portal vein. Using these procedures an attempt was made to determine whether the animals could tolerate glutamic acid better by the intraportal than by the peripheral intravenous route. Studies were also made in an attempt to relate the amino acid nitrogen and urea nitrogen content of the blood with the route of administration, and the production of vomiting.

Methods. Six adult male mongrel dogs were used in this study. Under intravenous nembutal anesthesia the abdomen was entered through a left rectus incision. After freeing the integument over the left half of the abdomen the left external and internal oblique muscles were resected. A rent was made in the transverse muscle and peritoneum through which the spleen was delivered. The hilus of the spleen was sutured to the margin of the peritoneum to prevent herniation of an abdominal viscus through it. The purpose of removing the external and internal oblique muscles was twofold: first, to allow more space for the implanted spleen; secondly, to prevent obstruction of the splenic circulation, either by scar tissue or by spasm of these muscles during an intrasplenic infusion. The abdomen was closed with interrupted cotton sutures.

In 3 animals, at the same time the spleen was transplanted, a small plastic tube† was sutured on the portal vein and brought to the outside through a small incision. The free end of the plastic tube was covered with sterile gauze and taped to the animal's side. This tube served the same purpose as the metal catheters used by London[4] in his angiostomy technic. Thus, by running a needle down this tube it was possible to infuse the glutamic acid solution into the portal vein in the unanesthetized animal without difficulty.

The glutamic acid solution used for all the infusions was prepared by adding 7 g of l +

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* A preliminary report of this work was given before the American Federation for Clinical Research, Chicago, October 30, 1947.

[1] Madden, S. C., Woods, R. R., Shull, F. W., Remintong, J. H., and Whipple, G. H., *J. Exp. Med.*, 1945, **81**, 439.

[2] Smyth, C. J., Levey, S., and Lasichak, A. G., *Am. J. Med. Sci.*, 1947, **214**, 281.

[3] Price, J. C., Waelsch, H., and Putnam, T. J., *J. Am. Med. Assn.*, 1943, **122**, 1153.

† The plastic tubing used was that supplied by the Baxter Company for their disposable infusion sets.

[4] London, E. S., Harvey Lectures, 1927-28, p. 208, Williams & Wilkins Co.

TABLE I.
Effect of Route of Administration of Glutamic Acid Solutions on the Production of Vomiting in Dogs.

Dog No.	Wt, kg	Glutamic acid admin.		Rate, ml/min	Reaction
		Route	Vol. received, ml		
1	11.8	I.V.[*]	150	10.0	Vomited
		I.S.[†]	200	8.0	0
		I.V.	145	7.2	Vomited
		I.S.	275	11.2	0
		I.V.	145	8.0	Vomited
		I.S.	285	9.5	0
		I.V.	150	8.8	Vomited
2	16.6	I.V.	80	7.5	Vomited
		I.S.	125	8.9	’’
		I.V.	58	8.3	’’
		I.S.	150	12.5	0
		I.S.	160	10.0	Vomited
		Intestinal vein	135	10.3	Vomited
3	15.4	I.V.	100	11.0	’’
		I.S.	175	8.0	’’
		I.V.	150	10.8	’’
4	12.2	I.V.	150	10.0	’’
		I.S.	315	10.5	’’
		I.V.	110	9.0	’’
		P.[‡]	200	10.1	’’
5	11.8	I.V.	95	9.5	’’
		I.S.	155	10.3	’’
		I.V.	100	12.2	’’
		I.V.	100	9.5	’’
		I.S.	160	8.0	’’
		P.	300	8.4	’’ after infusion stopped
6	11.4	I.V.	95	5.0	Vomited
		I.S.	175	5.0	’’
		P.	165	4.5	’’

[*] Intravenous.
[†] Intrasplenic.
[‡] Injection into the portal vein by the angiostomy technic.

glutamic acid (Merck)[‡] and 2 g of sodium bicarbonate to 500 ml of pyrogen-free saline. The solutions were sterilized by autoclaving.

Approximately 3 weeks after the transplantation of the spleen, the animals received the first intravenous infusion of glutamic acid. The solution was allowed to run into a leg vein at a uniform rate until the animal vomited. At this point the volume infused and the rate of infusion were recorded. Several days subsequent to the intravenous infusion the animal received an intrasplenic injection of glutamic acid. Since the transplanted spleen permitted the palpation of the gland, the needle could be easily directed into this organ. In addition, the intrasplenic position of the needle was confirmed by the aspiration of blood fluid. The solution was allowed to flow into the substance of the spleen at a uniform rate until the animal vomited. In some cases when approximately two times the intravenous dose was given into the spleen and the animal did not vomit, the intrasplenic infusion was discontinued because of danger of overhydration. After a few days

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[‡] We wish to thank Merck and Co., Inc., Rahway, N. J., for supplying the glutamic acid used in this study.

3 of the animals received a third infusion directly into the portal vein by the angiostomy technic, and the volume of fluid necessary to make the animal vomit was recorded. Some of the animals received more than one test involving a single method of infusion.

Results and discussion. A comparison of the tolerance of animals to glutamic acid administered either intravenously or directly into the portal system is presented in Table I. More glutamic acid could be administered into the portal system without the animals vomiting than could be given into the peripheral venous circulation. In a single case (Dog 2) the glutamic acid solution was injected into an intestinal vein isolated under local anesthesia and a greater tolerance was found for the amino acid when given in this manner than when administered into one of the leg veins.

Since there was no constant difference in either the blood amino acid nitrogen or the urea plus ammonia nitrogen dependent on the route of administration these data will not be presented. Also there was no level of blood amino acid nitrogen following the glutamic acid infusion which was uniformly associated with vomiting.

This work was initiated on the assumption that free glutamic acid could be partially destroyed or removed by passage through the liver. Thus if an infusion of this amino acid were permitted to pass first into the liver it should be better tolerated than if it were given into a peripheral vein. The present study supports this view. The vomiting which followed the intraportal infusions may signify that the capacity of the liver to remove this substance is exceeded. Friedberg and Greenberg[5] have reported the partition of amino acid nitrogen among the various tissues of the rat 15 minutes after the intravenous administration of glutamic acid. They found that the amino acid is slowly cleared from the plasma and at the same time the concentration of the amino acid nitrogen in the liver approached a control value. This could be interpreted as showing that free glutamic acid is rapidly being destroyed or conjugated in the liver.

Summary and conclusions. A method is presented by which the spleen may be readily transplanted subcutaneously in dogs and used for intraportal infusions.

Dogs could tolerate more glutamic acid solution without vomiting when it was given either intrasplenically or directly into the portal vein by using the angiostomy technic, as compared to the peripheral venous administration.

The increased tolerance to glutamic acid solution when given intraportally is attributed to the direct passage of these amino acids to the liver where they may be removed from the circulation.

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[5] Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, 1947, **168**, 411.

16830

Effect of Atmospheric Carbon Dioxide on Adrenal Cortical Hyperplasia and Associated Changes Due to Stress.

CLAUDE FORTIER.* (Introduced by H. Selye.)

From the Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal, Montreal, Canada.

By submitting animals to anoxia, under a high carbon dioxide partial pressure, Langley,[1] Hailman,[2] and their co-workers succeeded in preventing the activation of the adrenal

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* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

[1] Langley, L. L., Nims, L. F., Harvey, T. S., and Clarke, R. W., National Res. Counc. Div. Med. Sc., 1943, Rep. 108.

[2] Hailman, H. F., *Endocrinology*, 1944, **34**, 187.

TABLE I.
Effect on Organ Weights* of Exposure to Stress Under a 15% Partial Pressure of Carbon Dioxide.

Group	Treatment	Adrenals (mg)	Spleen (mg)	Thymus (mg)	Lymph nodes (mg)
I	None	9.3 ± 1.1	731.6 ± 41.9	167.6 ± 14	9.1 ± 1.1
II	Spinal cord transection + 15% CO_2	17.9 ± 2.5	472.9 ± 72.9	160.4 ± 9.6	5.7 ± 0.7
III	Immobilization + 15% CO_2	13.9 ± 1.7	274.6 ± 48.4	136.1 ± 8.9	6.4 ± 1.6
IV	Spinal cord transection + normal CO_2	11.6 ± 1.0	539.1 ± 102.8	127.5 ± 16.1	8.9 ± 1.6
V	Immobilization + normal CO_2	15.4 ± 2.9	414.0 ± 42.9	95.3 ± 13.8	7.2 ± 0.4
VI	15% CO_2 without other form of stress	16.1 ± 1.1	645.1 ± 250	149.0 ± 32.4	10.1 ± 1.1

* The mean organ weights are expressed per 100 g of final body weight.

cortex previously observed to follow acute or chronic exposure to decreased barometric pressure alone.[3-18] These investigators accordingly suggest that the stimulus responsible for the adrenal response during anoxia, is the alkaline shift of the acid-base balance which results from hyperventilation and the following acapnia.[19] The object of this study was to examine whether this mechanism, *i.e.* alkalosis, represents a necessary factor to the initiation of the alarm-reaction[20] or whether it is to be considered as but one form of non-specific stress.

Experimental. Forty-eight male albino rats of the Wistar strain weighing between 120 and 140 g at the onset of the experiment were subdivided into 6 groups of 8 animals. All experimental groups were fasted for the duration of the exposure. Group I received no treatment and served as a normal control group. Groups II and III were submitted respectively, under a 15% partial pressure of carbon dioxide, to 2 different forms of stress: spinal cord transection for Group II and immobilization on a board for Group III, both usually giving a well marked response within 24 hours.[21-23] Groups IV and V, which served as experimental controls, were exposed to the same types of stress under normal atmospheric conditions, while the animals of Group VI were subjected, without any additional alarming agent, to the elevated (15%) carbon dioxide tension.

A well ventilated decompression chamber

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[3] Armstrong, H. G., and Heim, J. W., *J. Aviat. Med.*, 1938, **9**, 92.

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[5] Evans, G., *Am. J. Physiol.*, 1934, **110**, 273.

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[7] Fitzgerald, O., *Arch. F. O. Ges. Physiol.*, 1939, **241**, 741.

[8] Giragossintz, G., and Sundstroem, E. S., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 432.

[9] Langley, L. L., Conference on factors producing hypertrophy of the adrenal cortex, Macy Foundation, 1942.

[10] Langley, L. L., *Fed. Proc.* 1943, **2**, 1.

[11] Langley, L. L., and Clarke, R. W., *Yale J. Biol. and Med.*, 1942, **14**, 529.

[12] Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. S., *J. Clin. Invest.*, 1942, **21**, 33. Medical Res. Council, London, Haemoglobin Committee. Spec. Rep. No. 72, 1923.

[13] Nims, L. F., Conference on factors producing hypertrophy of the adrenal cortex, Macy Foundation, 1942.

[14] Reynolds, O. E., and Phillips, N. E., *Am. J. Physiol.*, 1947, **151**, 147.

[15] Sundstroem, E. S., and Michaels, G., *Memoirs of the University of California*, 1942, **12**, 1.

[16] Tepperman, J. H., Engel, F. L., and Long, C. N. H., *Endocrinology*, 1943, **32**, 373.

[17] Tepperman, J. H., Tepperman, M., and Patton, B. W., *Endocrinology*, 1947, **41**, 356.

[18] Thorn, G. W., Jones, B. F., Lewis, R. A., Mitchell, E. R., and Koepf, G. F., *Am. J. Physiol.*, 1942, **137**, 606.

[19] Van Slyke, D. D., *J. Biol. Chem.*, 1921, **48**, 153.

[20] Selye, Hans, *Canad. Med. Assn. J.*, 1936, **34**, 706.

[21] Frank, J. D., *Endocrinology*, 1940, **27**, 447.

[22] Selye, Hans, *Brit. J. Exp. Pathol.*, 1936, **17**, 234.

[23] Selye, Hans, *Endocrinology*, 1937, **21**, 169.

3 of the animals received a third infusion directly into the portal vein by the angiostomy technic, and the volume of fluid necessary to make the animal vomit was recorded. Some of the animals received more than one test involving a single method of infusion.

Results and discussion. A comparison of the tolerance of animals to glutamic acid administered either intravenously or directly into the portal system is presented in Table I. More glutamic acid could be administered into the portal system without the animals vomiting than could be given into the peripheral venous circulation. In a single case (Dog 2) the glutamic acid solution was injected into an intestinal vein isolated under local anesthesia and a greater tolerance was found for the amino acid when given in this manner than when administered into one of the leg veins.

Since there was no constant difference in either the blood amino acid nitrogen or the urea plus ammonia nitrogen dependent on the route of administration these data will not be presented. Also there was no level of blood amino acid nitrogen following the glutamic acid infusion which was uniformly associated with vomiting.

This work was initiated on the assumption that free glutamic acid could be partially destroyed or removed by passage through the liver. Thus if an infusion of this amino acid were permitted to pass first into the liver it should be better tolerated than if it were given into a peripheral vein. The present study supports this view. The vomiting which followed the intraportal infusions may signify that the capacity of the liver to remove this substance is exceeded. Friedberg and Greenberg[5] have reported the partition of amino acid nitrogen among the various tissues of the rat 15 minutes after the intravenous administration of glutamic acid. They found that the amino acid is slowly cleared from the plasma and at the same time the concentration of the amino acid nitrogen in the liver approached a control value. This could be interpreted as showing that free glutamic acid is rapidly being destroyed or conjugated in the liver.

Summary and conclusions. A method is presented by which the spleen may be readily transplanted subcutaneously in dogs and used for intraportal infusions.

Dogs could tolerate more glutamic acid solution without vomiting when it was given either intrasplenically or directly into the portal vein by using the angiostomy technic, as compared to the peripheral venous administration.

The increased tolerance to glutamic acid solution when given intraportally is attributed to the direct passage of these amino acids to the liver where they may be removed from the circulation.

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[5] Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, 1947, **168**, 411.

16830

Effect of Atmospheric Carbon Dioxide on Adrenal Cortical Hyperplasia and Associated Changes Due to Stress.

CLAUDE FORTIER.* (Introduced by H. Selye.)

From the Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal, Montreal, Canada.

By submitting animals to anoxia, under a high carbon dioxide partial pressure, Langley,[1] Hailman,[2] and their co-workers succeeded in preventing the activation of the adrenal

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* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

[1] Langley, L. L., Nims, L. F., Harvey, T. S., and Clarke, R. W., National Res. Counc. Div. Med. Sc., 1943, Rep. 108.

[2] Hailman, H. F., *Endocrinology*, 1944, **34**, 187.

TABLE I.
Effect on Organ Weights* of Exposure to Stress Under a 15% Partial Pressure of Carbon Dioxide.

Group	Treatment	Adrenals (mg)	Spleen (mg)	Thymus (mg)	Lymph nodes (mg)
I	None	9.3 ± 1.1	731.6 ± 41.9	167.6 ± 14	9.1 ± 1.1
II	Spinal cord transection + 15% CO_2	17.9 ± 2.5	472.9 ± 72.9	160.4 ± 9.6	5.7 ± 0.7
III	Immobilization + 15% CO_2	13.9 ± 1.7	274.6 ± 48.4	136.1 ± 8.9	6.4 ± 1.6
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Experimental. Forty-eight male albino rats of the Wistar strain weighing between 120 and 140 g at the onset of the experiment were subdivided into 6 groups of 8 animals. All experimental groups were fasted for the duration of the exposure. Group I received no treatment and served as a normal control group. Groups II and III were submitted respectively, under a 15% partial pressure of carbon dioxide, to 2 different forms of stress: spinal cord transection for Group II and immobilization on a board for Group III, both usually giving a well marked response within 24 hours.[21-23] Groups IV and V, which served as experimental controls, were exposed to the same types of stress under normal atmospheric conditions, while the animals of Group VI were subjected, without any additional alarming agent, to the elevated (15%) carbon dioxide tension.

A well ventilated decompression chamber

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[3] Armstrong, H. G., and Heim, J. W., *J. Aviat. Med.*, 1938, **9**, 92.

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[7] Fitzgerald, O., *Arch. F. O. Ges. Physiol.*, 1939, **241**, 741.

[8] Giragossintz, G., and Sundstroem, E. S., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 432.

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[11] Langley, L. L., and Clarke, R. W., *Yale J. Biol. and Med.*, 1942, **14**, 529.

[12] Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. S., *J. Clin. Invest.*, 1942, **21**, 33. Medical Res. Council, London, Haemoglobin Committee. Spec. Rep. No. 72, 1923.

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[14] Reynolds, O. E., and Phillips, N. E., *Am. J. Physiol.*, 1947, **151**, 147.

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[17] Tepperman, J. H., Tepperman, M., and Patton, B. W., *Endocrinology*, 1947, **41**, 356.

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[19] Van Slyke, D. D., *J. Biol. Chem.*, 1921, **48**, 153.

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[21] Frank, J. D., *Endocrinology*, 1940, **27**, 447.

[22] Selye, Hans, *Brit. J. Exp. Pathol.*, 1936, **17**, 234.

[23] Selye, Hans, *Endocrinology*, 1937, **21**, 169.

TABLE II.
Probability That the Difference Between Mean Organ Weights Shown in Table I Might Have Occurred by Chance (P).*

Group	II-I	II-IV	III-I	III-V	IV-I	V-I	VI-I
Adrenals	$<.01$	$<.05$	$<.02$	$>.20$†	$>.10$†	$<.05$	$<.01$
Spleen	$<.01$	$>.20$†	$<.01$	$>.20$†	$>.10$†	$<.01$	$<.01$
Thymus	$>.20$†	$>.10$†	$<.01$	$<.05$	$<.05$	$<.05$	$<.03$
Lymph Nodes	$<.01$	$>.10$†	$<.02$	$<.05$	$<.02$	$>.10$†	$>.20$†

* From Fisher's table of T values.
† Difference not statistically significant.

of the R.C.A.F. standard model was used for maintaining a constant composition of the atmosphere, namely, CO_2, 15%; O_2, 19%; N_2, 66% at a pressure of 760 mm HG. and under normal hygrometric conditions. Samples of the gaseous mixture were analyzed at intervals by the Haldane method. The animals were killed after 38 hours by exposure and the adrenals, spleen, thymus and pelvic lymph nodes were weighed in the fresh state and histologically examined.

Results. The results are summarized in Table I. The probability of a chance occurrence of the differences in the means of the experimental groups (II, III and VI) from those of the normal control group (I) and the experimental control groups (IV and V), is indicated in Table II, in which we have taken as significant those differences for which the probability of occurrence by chance is not greater than 0.05. An increase in the weight of the adrenal glands is evident in both experimental and experimental control groups. In all but Group IV, this increase represents a significant deviation from the untreated control group. Conversely, the spleen, thymus and pelvic lymph nodes show in most groups, a significant decrease in weight which roughly parallels the increase in adrenal weight. The organ weights of Groups II and III, which were exposed to stress under a 15% pressure of carbon dioxide, are not significantly different from those of Groups IV and V which were submitted to the same damaging agents under normal atmospheric conditions. The adrenal hypertrophy and the splenic and thymic involution of Group VI which was exposed to 15% carbon dioxide without additional damaging agent, are significant. The histological examination of the organs revealed in all but Group I various degrees of lipid depletion in the cortex of the adrenals and of nuclear pyknosis in the lymphatic organs. These changes have been repeatedly described in connection with exposure to any non-specific damaging agent.[24]

Conclusion. Our results disprove the possibility that an alkaline shift of the acid-base balance is the necessary prerequisite for the development of the alarm-reaction, as the response of the organs (adrenals, spleen, thymus and lymph nodes) was of the same order in animals submitted to high and normal carbon dioxide tensions. The adrenal cortical hyperplasia as well as lymphatic tissue involution of the animals exposed, without other change in their environment, to a high carbon dioxide atmospheric tension, imply that this factor is in itself an alarming stimulus, perhaps through its action on the acid-base balance of the blood.[25]

This work was subsidized by a grant from the Commonwealth Fund of New York. The author is also indebted to Drs. L. Brouha, L. P. Dugal and A. Desmarais of the Institut d'Hygiène et de Biologie humaine, Université Laval, for the use of their decompression chamber.

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[24] Selye, Hans, *J. Clin. Endocrinol.*, 1946, **6**, 117.

[25] Waud, R. A., *Tr. Roy. Soc. Canada*, Sect. V, Biol. Sc., 1944, **38**, 103.

16831

The Role of Copper in Mammalian Pigmentation.

PETER FLESCH.* (Introduced by S. Rothman.)

From the Departments of Pharmacology and Medicine, Section of Dermatology, The University of Chicago.

Copper is an essential dietary factor for maintaining the color of fur in several species of mammals. Keil and Nelson[1] observed that black and piebald rats turned grey when fed on a diet deficient in copper. The original hair color could be restored within a few weeks by daily addition of 50 μg of copper to the diet. These observations were confirmed and extended to other species of mammals.[2-4] The depigmentation of copper-deficient rats and rabbits could not be cured by supplementing the diet with manganese, iron[4,5] or with vitamin B factors.[6] Copper was assumed to act as a catalyst of mammalian pigment formation[7] but there was no direct evidence to support this theory.

Experimental. The experiments which follow were carried out to elucidate the possible role of copper in the production of melanin. One set dealt with the effect of heavy metal catalysts and of inhibitors on melanin formation *in vitro*. In another series copper determinations were carried out on pigmented biological material.

The darkening of buffered solutions of dopa (1-dihydroxyphenylalanine, Hoffmann La Roche) was measured colorimetrically. All vessels and test tubes were washed with a 1:1 mixture of concentrated HCl and HNO_3 and rinsed 5 times with both distilled and double-distilled water. To avoid introducing traces of heavy metals, barbiturate buffer of pH 7.4 was used throughout and all solutions were made with double-distilled water. Each test tube contained 1 ml of 5:10,000 dopa solution, 1 ml heavy metal salt solution in a 10^{-4} to 10^{-6} M final concentration and 3 ml of the buffer. The test tubes were kept in the incubator at 37° and readings were made in the Klett-Summerson photoelectric colorimeter with filter KS-42 at hourly intervals. Cysteine hydrochloride (2×10^{-3} to 10^{-4} M), phenylthiourea (10^{-4} M) and aqueous extracts of isolated human epidermis[8] were used as inhibitors.

Copper was determined in hair and tissues. The hair was clipped from the animals, washed 5 times with chloroform for several days and then with acetone, dried, weighed and ashed at 500°C. The tissues were defatted according to Greenstein[9] and ashed. The ash was heated with 1 drop of concentrated HNO_3, dissolved in 6 N HCl and the copper determined by the diethyldithiocarbamate method.[9] Iron was estimated by the 2-2′ bipyridine method[10] and manganese by the catalytic method of Kun.[11] All determinations were carried out in duplicate and only data agreeing within 10% were accepted.

The melanin used for copper analysis was prepared from Harding-Passey melanomas of mice by digesting the ground tumor tissue

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* American Cancer Society Fellow, 1947-48.

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[3] Gorter, F. J., *Z. f. Vitaminforschung*, 1935, **4**, 277.

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[6] Free, A. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 371.

[7] Sarata, U., *Japan J. Med. Sci.*, II. Biochem., 1935, **3**, 79.

[8] Rothman, S., Krysa, H. F., and Smiljanic, A. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 208.

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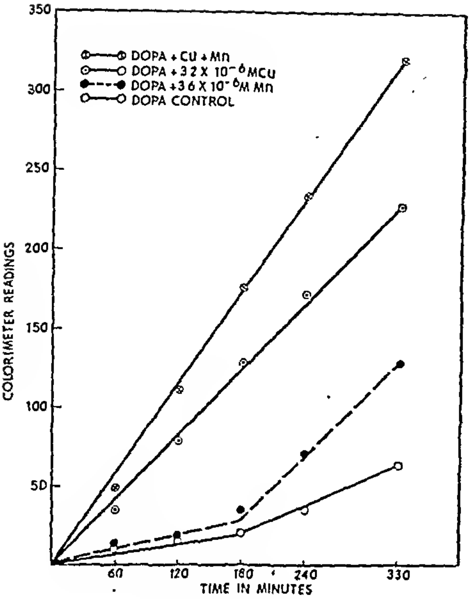

FIG. 1.

Catalytic effect of cupric and manganous ions on the autoxidation of dopa.

with pepsin-hydrochloric acid or with trypsin[12] for 4 weeks. The solution was changed at the end of 2 weeks. The digested tumor tissue was centrifuged and the supernatant fluid was dialyzed against double-distilled water for 7-10 days in the ice-box. The contents of the dialyzing bag were centrifuged, and the black precipitate was washed several times with double-distilled water and analyzed. In another series of experiments ground tumor tissue was kept overnight in 1% NaOH (5 ml/g wet tissue) at room temperature, then boiled for 30 minutes. The dissolved tissue was purified by dialysis, then dried for analysis.

Human epidermis from autopsy material was isolated by the heat method of Baumberger *et al.*,[13] or by treating the skin with 2 N KI solution.[14] Copper determinations were carried out on the defatted dried material.

Results. Catalytic activity of heavy metals on the autoxidation of dopa in vitro. The order of caralytic effect of the metals studied on the autoxidation of dopa was Cu>Co>Ni>Mn>Pb>Fe. Zn had very little effect, Mg and Hg none. The effect is additive (Fig. 1). In agreement with Bernheim[15] it was found that phenylthiourea which inhibits the tyrosine tyrosinase reaction *in vitro*[16,17] and pigmentation *in vivo*[18] had no effect on the autoxidation of dopa. In relatively high concentrations (10^{-4} M) it had only a slight inhibitory influence on the catalytic activity of cupric ion in the oxidation of dopa.

One mole of cupric ion was able to counteract the inhibition of the autoxidation of dopa produced by approximately 500 moles of cysteine (Fig. 2). Experiments with aqueous extracts of isolated epidermis confirmed the findings of Rothman and coworkers of an inhibitory factor in these extracts.[8] The factor was found to be heat stable and dialyzable and antagonized by cupric ion and by p-chloromercuribenzoic acid. This supports the previously advanced theory that the inhibition is due to sulfhydryl compounds.

Copper and iron determinations in hair. As suitable pigmented biological material, the hair of mottled rabbits, guinea pigs and rats

TABLE I.

Copper in μg/g in the Hair of Pigmented and White Areas of the Same Animal.

Animals	Copper content in μg/g hair	
8 rabbits	Grey or black: 35.4 (23.2-41.4)	White: 25.1 (9.3-35.8)
2 ”	Light brown: 22.5 (22.2-22.8)	White: 20.6 (20.2-20.9)
5 guinea pigs	Black: 26.2 (9.0-46.0)	White: 15.5 (1.0-47.1)
2 ” ”	Black: 16.4 (13.0-19.8)	Red-brown: 8.7 (6.6-10.8)
3 ” ”	Red-brown: 26.5 (18.5-35.5)	White: 26.6 (18.3-36.3)
4 rats	Black: 29.1 (13.3-38.9)	White: 23.5 (13.9-31.6)

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[12] Greenstein, J. P., Turner, F. C., and Jenrette. W. V., *J. Nat. Cancer Inst.*, 1940, **1**, 377.

[13] Baumberger, J. P., Suntzeff, V., and Cowdry, E. V., *J. Nat. Cancer Inst.*, 1942, **2**, 413.

[14] Felsher, Z., *J. Invest. Dermatol.*, 1947, **8**, 35.

[15] Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, 1942, **145**, 213.

[16] Paschkis, K. E., Cantarow, A., Hart, W. M., and Rakoff, A. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **57**, 37.

[17] DuBois, K. P., and Erway, W. F., *J. Biol. Chem.*, 1946, **165**, 711.

[18] Richter, C. P., and Clisby, K. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **48**, 684.

TABLE II.
Copper in Human Epidermis in μg/g Dry Fat-free Tissue After Separation of Epidermis from Corium.

White epidermis		Corium	Negro epidermis		Corium
No. 1	73.5	35.4	No. 1	50.2	15.2
No. 2	53.0	39.6	No. 2	53.5	24.4
No. 3	88.9	28.7	No. 3	51.9	11.1
No. 4	29.7	12.0	No. 4	49.4	6.2
No. 5	56.3	18.1			
No. 6	142.7	33.0			
No. 7	186.6	31.5			
No. 8	180.2	—			
No. 9	139.4	—			

TABLE III.
Copper Determinations in Harding-Passey Melanomas and in Melanin Prepared from the Tumor.

No.	Wet wt of tumor. g	Water content, %	Cu in μg/g dry fat-free tumor tissue	Cu in μg/g melanin
1	7.10	79.7	11.5	—
2	6.75	79.4	13.5	—
3	5.26	79.0	12.5	50.4
4	5.20	—	26.2	337.9
5	4.60	79.7	15.1	—
6	4.28	78.8	17.5	163.0
7	2.45	81.3	22.4	—
8	1.95	78.5	26.5	—
9	0.52	—	59.2	294.0

was chosen for copper determinations. By comparing white and colored hair from the same animal, individual variations in the copper content due to age, strain, and diet of the animals[19] were excluded. The results are presented in Table I.

The black or grey hair of 6 of the 8 rabbits, 6 of the 7 guinea pigs, and 2 of the 4 rats tested contained significantly more copper than the white or red brown hair of the same animals. No difference was found in the copper content of white and brown hair.

Determinations carried out on guinea pig hair showed that the iron content of the red-brown hair was considerably higher than that of the white hair of the same animal. The mean values in 6 animals were 35.4 μg/g (25.0–46.1) for red-brown hair and 15.4 μg/g (4.5–23.2) for white.

Copper determinations in isolated human epidermis. Isolated human epidermis obtained from autopsy material showed the same large individual variations in copper content as had the hair of animals. Preliminary experiments with Negro epidermis indicated a copper content in the same range as that found in white specimens, but not enough experimental material was available to allow making definite conclusions. The copper content of the epidermis invariably was higher than that of the corium. (Table II).

Copper determinations in Harding-Passey mouse melanomas. The tumor tested has been carried in this laboratory for one year in mice of the dba strain. Copper determinations were made on such tumors and on the kidneys and spleens in normal and tumor bearing animals. In the same animal the copper content of melanomas was below that of the kidneys (71.3 μg/g dry weight) and spleens (52 μg/g). Larger and older tumors had a lower copper content than recently implanted ones and all tumors had a relatively high water content.

The melanin pigment prepared from the melanomas by boiling with NaOH, contained 4 to 13 times more copper than the tumor. (Table III) In the amounts used in the preparation, the NaOH could have accounted for only about 5% of the copper found in the melanin, a percentage within the experimental error of the copper determination. On the other hand, the melanin pigment prepared by peptic and tryptic digestion yielded a copper content 20 to 95 times higher than the tumor tissue. The difference may have been due to more complete hydrolysis of the tumor tissue, but since both the pepsin and trypsin used had relatively high copper contents, the possibility could not be excluded that some copper in the pigment prepared by digestion was of extraneous origin. These data are therefore not presented. On the other hand, the manganese content of the tumor (57.3 μg/100 g dry fat free tissue) was practically identical with that of the pigment (63.2 μg/100 g) derived from it. Values obtained with liver and kidney were in the same range.

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[19] Cunningham, I. J., *Biochem. J.*. 1931. 25, 1267.

Discussion. The catalytic influence of cupric ions on the autoxidation of dopa has been described by several authors.[7,19] No comparative experiments with other heavy metallic salts have been reported. Sarata[7] observed that copper sulfate exerted a catalytic effect only in a certain range of concentration. In this laboratory the apparent inhibition by relatively high concentrations of copper sulfate, described by Sarata, was found to be due to the use in his experiments of unbuffered solutions; in relatively large amounts. copper sulfate lowered the pH below the neutral range. In some of our experiments, involving the use of buffered solutions, copper sulfate in concentrations which Sarata had found to be inhibitory (5 μg/ml) showed a more powerful catalytic action than in lower concentrations. Copper determinations on the hair of mammals have been made by several authors. In two papers its concentration was reported to be higher in black hair than in white hair from the same animal,[7,19] while in others no such difference was found.[20,21] However, the determinations were on a very limited number of animals. Melanogenesis takes place in the germinative epithelial layer of the hair and even if copper is involved, it may not ascend with the pigment in the growing hair. The higher iron values in red-brown guinea pig hair than in white hair are of interest in view of the fact that an iron-containing red pigment has been isolated from human red hair.[22] The literature has no data on the copper content of isolated human epidermis. Relative values for whole skin, expressed in relative intensities of spectral lines, showed the same large individual variations as observed in our experiments.[23] In bovine epidermis, Cunningham[19] found much larger values than in the dermis of the same animal. He suggested that the skin was an important organ for copper excretion. Since the pigment constitutes such a small fraction of the entire epidermis, a difference in the copper content of pigmented and non-pigmented skin may not be detectable. This would explain the relatively low copper values in the epidermis of the Negro. The low copper values of melanotic tumors agree with the data of other authors.[19,24,25] However, the finding

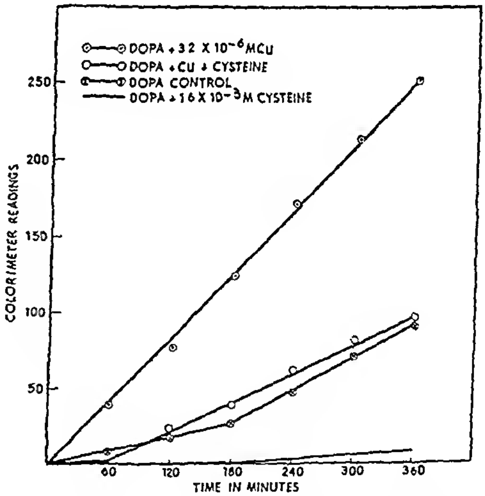

FIG. 2.

Suppression, by cupric ion, of the inhibitory effect of cysteine hydrochloride on the autoxidation of dopa.

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[20] Cohen, G. N., *Trav. membres soc. chim. biol.*, 1941, **23**, 1504.

[21] Saccardi, P., and Giuliani, G., *Biochem. terap. sper.*, 1935, **22**, 169.

[22] Flesch, P., and Rothman, S., *J. Invest. Dermatol.*, 1945, **6**, 257.

[23] McCardle, R. C., Engman, M. F., Jr., and Engman, F. R., Sr., *Arch. Dermatol. Syphilol.*, 1941, **44**, 429.

[24] Greenstein, J. P., Werne, J., Eschenbrenner, A. B., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, 1944, **5**, 55.

that the isolated pigmented part of the tumor is strikingly high in copper concentration strongly favors the theory that copper acts as a local catalyst in pigment formation, probably as part of an oxidative enzyme, possibly tyrosinase which has recently been demonstrated in melanotic tumors of mice.[26,27] Mammalian tyrosinase contains significant amounts of copper and can be inhibited by substances which combine with this metal.[28] Copper apparently also forms a metallo-organic complex with dopa[29] and may remain attached to the melanin molecule. In this connection it is of interest to note that the ash of octopus ink contains as much as 2% copper.[30]

We advance the theory that copper may promote pigmentation, not only by direct action on the substrate, but also indirectly by oxidizing sulfhydryl groups which inhibit pigmentation *in vitro*,[31] and probably also *in vivo*.[8,32,33] Greenstein *et al.* have indicated that melanin in mouse melanomas is attached to the rest of the pseudoglobulin molecule in the vicinity of sulfur containing amino acids.[12] Our finding that copper, too, is closely linked to the pigment suggests an interaction between copper and sulfhydryl compounds.

Summary 1. Autoxidation of dopa *in vitro* was more strongly catalyzed by cupric ions than by any of the other heavy metallic salts investigated. The order of catalytic activity was Cu>Co>Ni>Mn>Pb>Fe.

2. Black and grey hair of rabbits, guinea pigs and rats generally, but not always, contained more copper than white hair of the same individual. In guinea pigs, red-brown hair contained more iron than white hair.

3. Analysis of the separated layers of human skin showed a considerably higher copper content in the epidermis than in the corium.

4. Melanin from mouse melanomas had a copper content 4 to 13 times higher than the tumor from which it was prepared.

5. The theory is advanced that copper plays a role as a local catalyst in mammalian pigmentation.

The author wishes to thank Dr. Stephen Rothman for his interest and assistance in the present work.

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[25] Hahn, P. F., and Fairman, E., *J. Biol. Chem.*, 1936, **113**, 161.

[26] Hogeboom, G. H., and Adams, M. H., *J. Biol. Chem.*, 1942, **145**, 273.

[27] Greenstein, J. P., and Algire, G. H., *J. Nat. Cancer Inst.*, 1944, **5**, 35.

[28] Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H., *Fed. Proc.*, 1948, **7**, 167.

[29] Eichholtz, F., and Birch-Hirschfeld, A., *Arch. f. exp. Path. u. Pharmakol*, 1933, **170**, 271.

[30] Guiliani, G., *Ann. Chim. farm.*, 1938, 61.

[31] Figge, F. H. J., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 269.

[32] Ginsburg, B., *Genetics*, 1944, **29**, 176.

[33] Schaaf, F., *Arch. f. Dermatol. u. Syph.*, 1938, **177**, 646.

16832 P

An Antidiuretic Substance in the Blood of Normal and Adrenalectomized Rats.*

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In adrenal insufficiency the diuretic response to water is greatly reduced or absent (reviewed by[1]). An attempt was made to see if this was due to the accumulation of anti-

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[1] Gaunt, R., *J. Clinical Endocrinology*, 1946, **6**, 595.

TABLE I.

Showing Mean Effect on Water Diuresis Produced by *Various Types of Blood* Serum.

Series	Type of serum tested: (1 cc sample)	No. *animals*	% water excreted at indicated min.			Urine chlorides	
			30	*60*	*90* ± S.E.*	mg/cc as NaCl	Total 3 hr
1	Normal—fresh	17	4	15	35 ± 3.56	1.06	11.15
2	Adx. 3-7 days—fresh	26	2	10	26 ± 2.22	1.14	11.82
3	Adx. 9-12 ” ”	10	1	6	20 ± 4.03	1.29	13.23
4	Normal—drawn 12 hr	16	20	45	66 ± 4.97	0.45	5.56
5	Adx.— ” 12 ”	5	9	32	51	0.55	7.07
6	Normal saline (no serum)	12	19	45	64 ± 3.67	0.43	6.21

$$* \text{S.E.} = \pm \sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$$

diuretic substances (called ADS) in body fluids. The use of a modified Heller and Urban[2] technic revealed that the serum of normal animals contained a consistently detectable amount of labile ADS and that its concentration increased after adrenalectomy.

Methods. In testing samples of blood serum for their antidiuretic content, male rats weighing approximately 200 g were fasted but allowed water for 18 hours. Two doses of water, 3 cc per 100 sq cm of body surface, warmed to body temperature, were then administered at hourly intervals to animals in individual metabolism cages. For a 200 g rat each dose was 9 cc. One hour after the administration of the second dose of water the urine was measured. Any rat which, during the 2 hour hydration period, had a urine excretion rate 50% more or less than the mean of the groups of 6 was discarded. This necessitated the elimination of 6%. The rats with a normal diuretic response were then placed over clean funnels, the test material was injected intraperitoneally, and a third dose of water was administered. Following the third dose of water the urine output was measured at 30 minute intervals for 3 hours. The water excreted was calculated as the per cent of the total water given (3 doses) minus that excreted prior to injection of test material. It was found that this method, designed not so much to equalize hydration, as to establish a high diuretic rate, permitted better determinations of ADS than the ordinary Burn assay procedure. The most consistent differences between groups occurred at 90 minutes and figures beyond that time are not tabulated here.

To obtain serum, blood was taken directly from the heart without anticoagulant after induction of ether anesthesia. The blood was quickly centrifuged, serum decanted, and immediately injected into test animals unless otherwise specified.

Results. As seen in Table I, Ser. 1, the injection of 1 cc of serum from normal animals depressed urine volume and increased chloride excretion. If the decanted serum samples were allowed to stand at 5 - 9°C for 12 hours the antidiuretic and chloruretic activity was lost (Ser. 4). The serum of animals adrenalectomized from 3 to 7 days had higher antidiuretic and somewhat higher chloruretic activity than that of normal animals (Ser. 2). Again most of the activity was lost in aged refrigerated serum (Ser. 5). Greater amounts of ADS appeared in animals adrenalectomized for 9 - 12 days (Ser. 3). For the first 18 hours after adrenalectomy rats show a normal diuretic response to small (5 cc per 100 g) water loads but not to larger ones.[1] It was found (Table II) that 5 milliunits of Pitressin[†] was more effective at 18 hours after adrenalectomy than in dummy-operated controls.

Discussion. While antidiuretic substances have been detected in body fluids in various conditions, results have been in general either

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[2] Heller, H., and Urban, F. F., *J. Physiol.*, 1935, **85**, 502.

[†] Supplied by Dr. D. A. McGinty, Parke, Davis and Company.

TABLE II.
Water Excretion in Rats Receiving Water (5 cc/100 g) and 5 Milliunits Pitressin.

	No. animals	% excreted at following min. 60	120	180 ± S.E.
Dummy-operated	12	5.5	47.5	79.9 ± 3.52
Adx. 18 hr	12	4.1	27.6	55.0 ± 4.50

scattered, inconsistent or controversial.[3-6] The test used here apparently reveals the presence of ADS consistently in normal rat serum. This method has been used by Dr. C. W. Lloyd of Syracuse University Medical College and yielded similar results (unpublished) both in normal human subjects and Addisonian patients. While our data is consistent with the hypothesis that the circulating ADS is of posterior pituitary origin, critical evidence on its site of origin and precise quantitative information regarding its activity are being sought.

Although it is obvious that the greater accumulation of ADS may account in part for the failure of water diuresis in adrenalectomized animals, a factor of equal importance may be the increased sensitivity to—rather than the increased amounts of—ADS. This is indicated by the observed augmented sensitivity to Pitressin after adrenalectomy. Thus, normal water excretion may be a consequence of the balanced interaction of diuretic cortical hormones and ADS of pituitary or other origin (concept of Corey and Britton[7]). After adrenalectomy, the ADS, present in increased amounts, could act unchecked by its normal antagonist. Such interpretations are consistent with recent studies on renal function in salt-treated adrenalectomized rats, which indicate that the failure of water excretion is due primarily to an accelerated tubular reabsorption (Lotspeich[8]). Although a decreased glomerular filtration may be observed in untreated adrenalectomized rats, it will not account for the failure of water excretion at moderately high water loads.[9] If the main factor involved is an active ADS, possibly of posterior pituitary origin, its expected action would be on renal tubular reabsorption. The problem, however, is a complex one because extra-renal factors have also been clearly demonstrated.[1,10]

Summary. The blood serum of normal rats contains a labile antidiuretic and chloruretic substance(s?) which increases in amount after adrenalectomy.

Addendum. Since the above was written additional work, in collaboration with Drs. W. R. Boss and C. M. Osborn, has established that: (1) the ADS in fresh normal rat serum does not affect glomerular filtration, as measured by creatinine clearance, and presumably therefore acts by increasing the renal tubular reabsorption of water; and (2) that no ADS is detectable in the blood of hypophysectomized rats.

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[3] Walker, A. M., *Am. J. Physiol.*, 1939, **127**, 519.

[4] Martin, S. J., Herrlich, H. C., and Fazekas, J. F., *Am. J. Physiol.*, 1939, **127**, 51.

[5] Ham, G. C., and Landis, E. M., *J. Clin. Invest.*, 1942, **21**, 455.

[6] Hare, K., Hickey, R. C., and Hare, R. S., *Am. J. Physiol.*, 1941, **134**, 240.

[7] Corey, E. L., and Britton, S. W., *Am. J. Physiol.*, 1941, **133**, 511.

[8] Lotspeich, W. D., *Fed. Proc.*, 1948, **7**, 74; and additional data by personal communication.

[9] Unpublished work of Drs. W. R. Boss and J. H. Birnie of this laboratory.

[10] Birnie, J. H., Eversole, W. J., and Gaunt, R., *Endocrinology*, 1948, **42**, 412.

16833

Glucose Tolerance in Decerebrated Rats After Relatively Long Survival.

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It has been amply demonstrated in acute experiments on rabbits and cats that piqûre or decerebration at the pontile level gives rise within less than an hour to hyperglycemia and glycosuria, and that the increase in sugar is usually maintained for 3 or 4 hours, but may last as long as 9 hours. Claude Bernard[1,2] observed that piqûre was most effective when done bilaterally at levels between tthe emergence of the vagus and acoustic nerves. Donhoffer and Macleod[3] demonstrated that hyperglycemia occurred consistently only when decerebration was done at the pontile level, and they were of the opinion that the diabetogenic center probably was situated in the tegmentum pontis. Brooks[4] presented evidence that the center is situated in the floor of the IVth ventricle just caudal to the middle of the brachium pontis and very close to the vasomotor center.

According to Donhoffer and Macleod,[3] midbrain decerebration leads to little or no hyperglycemia, an observation in accord with those of Olmsted and Logan,[5] Bazett, Tychowski and Crowell,[6] Peterson,[7] and Noltie.[8] On the other hand, a high and well sustained hyperglycemic response to midbrain decerebration has been noted by Anderson *et al.*[9] and Evans, Tsai and Young.[10]

The medulla oblongata also has been subjected to piqûre or other lesion in an effort to locate a diabetogenic center. Brugsch, Dresel and Lewey[11] came to the conclusion that the dorsal nucleus of the vagus was the center in point. On the other hand, Hiller[12] and Hiller and Tannenbaum[13] found relatively little increase in blood sugar following damage to this nucleus, and they contended that no blood-sugar-raising (or diabetogenic) center, as such, exists in the central nervous system, but their view depends on what definition the term "center" is given. According to Donhoffer and Macleod[3] and Macleod,[14] only a slight or moderate degree of hyperglycemia ensues after injury to the medulla oblongata.

The rat has been little used in experiments on decerebration-induced or piqûre-induced hyperglycemia. Bell, Horne and Magee[15]

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* With the technical assistance of Mrs. Ann Alvey, Mr. Vivian R. Loving, and Mr. William H. Good.

This work was begun in the Department of Physiology at Johns Hopkins University Medical School. The authors wish to thank Dr. Philip Bard and Dr. Reginald B. Bromiley for their invaluable help.

[1] Bernard, C., *Compt. rend. d. séances et mém. de la Soc. de biol.*, 1849, **1**, 14, 60.

[2] Bernard, C., *Leçons sur la physiologie et la pathologie du système nerveux*, vol. 1, J.-B. Baillière et fils, Paris, 1858, pp. 397-447.

[3] Donhoffer, C., and Macleod, J. J. R., *Proc. Roy. Soc. s.B.*, 1932, **110**, 125.

[4] Brooks, C. McC., *Am. J. Physiol.*, 1931, **99**, 64.

[5] Olmsted, J. M. D., and Logan, H. D., *Am. J. Physiol.*, 1923, **66**, 437.

[6] Bazett, H. C., Tychowski, W. Z., and Crowell, C., PROC. SOC. EXP. BIOL. AND MED., 1925, **22**, 39.

[7] Peterson, J. M., Ph.D. Thesis, University of Aberdeen, 1933 (cited by Macleod.[14])

[8] Noltie, H. R., *Quart. J. Exp. Physiol.*, 1938, **28**, 99.

[9] Anderson, I. A., Cleghorn, R. A., Macleod, J. J. R., and Peterson, J. M., *J. Physiol.*, 1931, **71**, 391.

[10] Evans, C. L., Tsai, C., and Young, F. G., *J. Physiol.*, 1931, **73**, 67, 81.

[11] Brugsch, T., Dresel, K., and Lewey, F. H., *Z. f. exp. Path. u. Therap.*, 1920, **21**, 358.

[12] Hiller, F., *Münch. med. Wchnschr.*, 1930, **1**, 836.

[13] Hiller, F., and Tannenbaum, A., *Arch. Neurol. and Psychiat.*, 1929, **22**, 901.

[14] Macleod, J. J. R., *Bull. Johns Hopkins Hosp.*, 1934, **54**, 79.

[15] Bell, D. J., Horne, E. A., and Magee, H. E., *J. Physiol.*, 1933, **78**, 196.

TABLE I.
Blood Sugar Values Within Four Hours After Pontile Decerebration.*

	Blood sugar (mg %)			
	Preoperative	Postoperative		
Rat No.	(under anesthesia)	Immediate	1 hr	4 hr
Decerebrated rats				
651	—	—	209	140
650	74	51	208	219
654	84	68	64	123
655	80	155	118	196
656	87	142	218	99
Control: partially decorticated rats				
507	—	—	86	84
300	59	86	86	78
301	77	156	95	58
302	70	155	87	72

* The extent of the lesions was verified on gross examination post mortem.

found that pontile decerebration caused no increase in blood sugar in fasted rats or in those on a well balanced diet, but did occur over a 2-hour period if they had been fed a diet rich in carbohydrates. They were unable to elicit hyperglycemia by midbrain decerebration.

All the decerebration experiments referred to were acute ones, the animals generally surviving not more than a day or two. It is the purpose of this communication to describe the effects of decerebration on the glucose tolerance of rats which survived the operation for from 4 to 20 days.

Methods. Young adult female rats of the Sprague-Dawley strain were used in this study. All had had access to food up to the time of operation. Sodium pentobarbital, 2.5 mg per 100 g body weight intraperitoneally, was the general anesthetic employed. Novocaine (2.0% solution) was used to infiltrate the scalp immediately before opening the skull. Just preceding the operation, 0.05 cc of calcium gluconate per 100 g body weight was given intravenously to facilitate clotting. Decerebration was done at pontile and midbrain levels. Using the dorsal approach, wedges of brain tissue were removed by suction, and an incision then made downward to the base of the skull.

There were two objectives in this study: a) to determine the blood sugar level within a short time after decerebration, and b) to study glucose tolerance after the lapse of several days.

a. In one series of rats, blood sugar levels were determined at the following intervals: 1) preoperatively, just after the animals had been anesthetized, 2) immediately following operation, 3) one hour after operation, and 4) 4 hours after operation.

b. In the other series the animals were given 10 cc of 10% glucose in saline parenterally 6 hours after decerebration. Twenty-four hours postoperatively they were placed on a regimen consisting of 10 cc liquid diet by stomach tube twice daily and 10 cc of 10% glucose in saline and 2500 units penicillin parenterally once a day. From the start they were kept in an incubator at a temperature of 27°C, and under these conditions their rectal temperature was usually 33 to 34°C. Serving as controls were 3 rats, one of which was partially decorticated, one hemidecerebrated, and one normal. These were kept on the same regimen as the experimental animals.

The glucose tolerance test was done on the animals at various times from the 3d to the 23d day after operation. For 17 hours before the test the animals were fasted; they also received no water until one hour before the test, when they were given 10 cc of physiologic solution of sodium chloride by the subcutaneous route. Just before the test was begun, the animals were lightly anesthetized by sodium pentobarbital 2.5 mg per 100 g

TABLE II.
Data on Glucose Tolerance and Autopsy Findings in Decerebrated Female Rats and Controls.

			Postoperative data									Autopsy data	
					Glucose tolerance (blood sugar in mg %)								
Rat No.	Wt (g)	Operation	Day	Wt (g)	Fast.	30 min.	60 min.	90 min.	120 min.	Survival (days)	Died (D) or sacrificed (S)	Wt (g)	
						Experimental							
343	174	Pontile Decerebration	4	158	120	—	278	398	456	18	D	157	Virtually complete destruction of pons to lower level of trapezoid body.
323	192	Pontile Decerebration	3	205	141	336	442	464	480	4	D	203	Decerebration at midpontile level grossly; microscopic examination not done.
312	187	Pontile Decerebration	7	172	108	117	380	440	504	14	S	169	Virtually complete destruction to lower level of pons.
336	140	Pontile Decerebration	3 6	138 141	84 97	285 247	271 300	— 305	— 326	9	D	126	Complete destruction of pons to level of 7th nerve.
309	216	Midbrain Decerebration	5	196	85.	304	420	170	140	20	S	171	Softening of tissue of rostral midbrain equivalent to midbrain decerebration.
						Control							
351	153	Partial Decortication	1 23	143 136	85 >35	111 105	141 140	— 136	— 136	23	S	136	Brain stem intact except superficial softening of dorsal thalamus bilaterally and dorsal epithalamus at one level.
304	169	Pontile hemidecerebration	7 13	134 128	101 79	222 162	204 88	224 80	242 85	13	S	128	Lateral 1/3 to 3/4 of brain stem destroyed unilaterally to lower level of trapezoid body.
318	175	None	5	170	67	193	243	248	229	18	S	165	No histologic changes observed.
43 normal rats (avg)			—	182	76	154	182	179	183	—	—	—	—

body weight. They were then placed in an animal holder with the tail resting on a warm plate. Blood was collected from the tip of the tail. After the fasting blood sample was taken, 5 cc of 20% glucose solution per 100 g body weight were given by stomach tube. Blood samples were taken every 15 minutes for 2 hours. During the glucose tolerance test the animals were quiet, and the rectal temperatures of the decerebrated animals were between 33° and 35°C. Over the period of several weeks during which these tests were carried out, glucose tolerance was done also on 43 normal female rats of the same age. For blood sugar determinations the micro-method of Haslewood and Strookman[16] was used.

At autopsy the brains were fixed in 10% formalin. Blocks were frozen and serially sectioned at 45 microns by the method described by Marshall,[17] and an average of 50 sections per case were stained, one-half by cresyl violet and the other half by a modified Weil myelin sheath method.

Results. a. Blood sugar values within 4 hours after pontile decerebration are indicated in Table I. In neither the decerebrated nor the partially decorticated animals was there an elevation of blood sugar following anesthetization. Immediately after operation, which required about 30 minutes, the blood sugar was somewhat elevated in about half the experimental and control groups. Subsequently there was hyperglycemia in the experimental animals, but not in the controls.

b. Glucose tolerance in rats of longer survival is shown in Table II. The studies were made on these animals from the 3d to the 7th day after operation. The decerebrated rats showed a fasting blood sugar which tended to be normal, but had a decreased tolerance for fed glucose, the blood sugar rising to levels between 450 to 500 mg % at the end of the second hour, except in one animal (rat 336) in which it was 326 mg %. The fifth rat in this series was subjected to decerebration at the midbrain level; it also showed a diminished tolerance for fed glucose, but the highest peak in the blood sugar curve appeared after one hour, and by the second hour the blood sugar level approached normal. The partially decorticated and hemidecerebrated control rats showed glucose tolerance curves which were in the normal range when comparison is made (1) with the values in an unoperated animal (rat 318) subjected to the same regimen as the experimental animals, and (2) with the average values of 43 normal rats which had been fed *ad libitum.*

In order to rule out extraneous factors affecting blood sugar, such as the handling of the decerebrated animal during the glucose tolerance test, a sham test was carried out; it differed from the regular test only in that water rather than glucose solution was given by stomach tube. That the factor of handling was of no import is indicated by the observation that blood sugar values prior to the giving of the water and every 15 minutes thereafter for 2 hours were found to range between 94 and 119 mg%.

Discussion. The experiments herein described differ from those previously reported in that the blood sugar studies were done on the 3d to the 7th day after decerebration. The tests were performed while the animals were in a "healthy state," as indicated by the length of the subsequent survival periods, which in one case was as long as 20 days. It will be necessary to repeat the work on decerebrate animals of longer survival before the duration of the disturbance in carbohydrate metabolism can be determined.

Another aspect of the work was the determination of blood sugar values during the few hours after decerebration. Hyperglycemia of moderate degree over about 4 hours occurred consistently in animals which had had access to food up to the time of operation. This observation differs from that of Bell, Horne and Magee, previously referred to, who found that hyperglycemia occurred only in animals fed a rich carbohydrate diet: ours had been on a standard stock diet. Thus, it would appear that the rat is no different from the rabbit and cat so far as the induction of hyperglycemia by decerebration is concerned.

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[16] Haslewood, G. A. D., and Strookman, T. A., *Biochem. J.*, 1939, **33**, 920.

[17] Marshall, W. H., *Stain Technol.*, 1940, **15**, 133.

This study confirms the well authenticated observation that disordered carbohydrate metabolism is most striking when decerebration is done at the pontile level. Unilateral piqûre of the floor of the IVth ventricle has been reported by several workers, including Claude Bernard, to give rise to transient hyperglycemia, but in one of our control animals (rat 304), in which the pons and midbrain were destroyed unilaterally up to the midline, the glucose tolerance was normal.

Under autopsy data in Table II, only the lower levels of the lesions are indicated, for these are regarded as the significant ones. At operation, considerable brain tissue was removed in order to be sure that decerebration was complete. In each of the animals decerebrated at the pontile level, autopsy studies disclosed either softening or absence of the midbrain, the epithalamus, the superior cerebrum, the hippocampus, and part or all of the thalamus, bilaterally. Free from lesions were the pituitary gland, hypothalamus, subthalamus, and lenticular nuclei, though in one instance (rat 343) the superior part of the hypothalamus at the infundibular level was softened. In the animal listed as having been subjected to midbrain decerebration (rat 309), the original intent was to do a decortication and have the animal serve as a control, but microscopic examination of serial sections of the brain disclosed destruction of the posterior thalamus, the most superior part of the hypothalamus, the pretectal region, the inferior colliculi, and the medial geniculate bodies, with softening of the lateral two-thirds of the rostral midbrain bilaterally.

Summary. Rats decerebrated at the pontile level exhibited a markedly elevated glucose tolerance curve 3 to 7 days after operation. One subjected to midbrain decerebration showed a shallower and less sustained curve. Hyperglycemia occurred in rats during the first few hours after pontile decerebration, which is in confirmation of previously published results on rabbits and cats.

16834 P

Effect of an Analogue of DDT on Experimental Murine Typhus.

Florence K. FitzPatrick. (Introduced by L. Earle Arnow.)

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In a search for rickettsiostatic agents that might be more effective than para aminobenzoic acid (PABA), a large group of miscellaneous substances has been tested in mice infected with murine typhus. The results obtained with one of these, 1,1,1-trichloro-2, 2-bis(para-nitrophenyl) ethane, a nitro analogue of DDT, seem to warrant a brief report at this time.* This compound, which will hereafter be referred to as the nitro analogue, was being tested in mice by Kikuth in Germany at the time of the surrender. Nothing is known of his experiments except his notation that the compound was more effective against murine typhus than was methylene blue, and that it was of low toxicity.[1]

We have been successful in treating mice infected with murine typhus with material prepared in this laboratory.† Mice of the

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* This substance was named Nitrogesarol by the Germans, a term which would erroneously lead to the belief that it was obtained as the result of the nitration of DDT.

[1] Publication Board Reports, Dept. of Commerce, Report No. 248, p. 63.

† We are indebted to Dr. E. J. Cragoe, Jr., of the Organic Chemistry Department for the preparation of this compound.

TABLE I.
Comparison of Nitro Analogue at 0.5% and PABA at 2.0% Levels.[*]

	Nitro analogue		PABA		Controls	
	No. of mice dead on day					
Exp. No.	7	12	7	12	7	8
1	0/6 [†]	2/6	0/6	4/6	3/6	6/6
2	1/14	4/14	3/14	14/14	12/14	14/14
3	1/6	6/6	1/6	4/6	5/6	6/6
4	0/9	3/9	0/9	5/9	7/9	9/9
5	0/6	4/6	0/6	6/6	6/6	—
6	0/6	1/6	0/6	4/6	9/9	—
7	0/6	6/6	0/6	4/6	6/6	—
Totals	2/53	26/53	4/53	41/53	48/56	56/56

[*] Levels of 0.5 and 2.0% in the diet represent average drug consumptions of 10 and 40 mg, respectively, per mouse per day.

[†] Numerator denotes number of mice dead; denominator, number of mice used.

dba strain[2] weighing from 11 to 15 g were infected by the intraperitoneal route with 0.25 ml of a 6 to 10% brain suspension that had been prepared from infected brains kept frozen at -60°C. Treated mice received the drug, which was incorporated in ground Rockland chow for 7 days, beginning at the time of infection. Surviving treated mice were observed until death or recovery had occurred. Most untreated controls were dead by the seventh day. Table I summarizes experiments in which the nitro analogue and PABA were compared. It reveals that a high rate of survival during the period of drug ingestion was obtained with both agents, though the level of drug fed in the case of the nitro analogue was only equal to a quarter of the PABA required to achieve the same result. When the drugs were withdrawn on the 7th day there were more delayed deaths in the PABA-treated mice.

In preliminary toxicity tests no untoward symptoms were observed in 20 mice for a week following a single oral dose of 5 g/kg of the compound suspended in tragacanth.

In contrast with the results obtained with its nitro analogue, DDT was found to be highly toxic at levels of 0.4 mg/mouse/day and higher. At a concentration of 0.2 mg/mouse/day, a level tolerated by the mice, no rickettsiacidal activity was observed.

Summary. The nitro analogue of DDT when given in the diet for 7 days to mice infected with murine typhus resulted in a degree of survival on the 7th day equal to that obtained when 4 times the amount of PABA was administered. After the 7th day, when the drug was withdrawn, more PABA-treated mice succumbed, indicating that the nitro analogue is a better rickettsiacidal agent. The oral toxicity of the compound for mice appears to be of a low order.

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[2] Moragues, V., and Pinkerton, H., *J. Exp. Med.*, 1944, 79, 35.

16835

Effect of Dibenamine on Renal Function.*

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Clinical interest[1-3] in Dibenamine (N,N-dibenzyl-β-chloroethylamine) has made a full understanding of its toxicity essential. Previous reports from his laboratory[4,5] indicate two major types of toxicity: (1) central nervous system stimulation, particularly when the drug is administered rapidly by the intravenous route, and (2) local tissue damage after subcutaneous or intramuscular administration. Before Dibenamine was made available for clinical trial, a histological study of the organs of rats receiving maximum tolerated or lethal doses for protracted periods was undertaken;[6] this study failed to reveal any renal damage even in animals dying from chronic Dibenamine administration.

The recent report of Ogden[7] that Dibenamine produced renal failure in one dog has questioned the safety of this agent. The present work was undertaken in an effort to evaluate more exactly the effect of Dibenamine on renal function.

Methods. Experiments were carried out on 5 female dogs weighing between 10 and 15 kg which were trained to lie quietly with only loose restraint during the collection periods. Glomerular filtration was determined by creatinine clearance and renal plasma flow by sodium p-aminohippurate (PAH) clearance. Approximately constant plasma levels of creatinine and PAH were maintained by the continuous infusion (about 8 ml/min) of 5% glucose solution containing creatinine and PAH. The plasma creatinine level was maintained at about 10 mg % while the PAH level was usually below 2 mg %. Bladder urine was collected through a rubber mushroom catheter, and at the end of each 10-minute collection period the bladder was washed with distilled water. Blood samples were taken at the midpoint of each urine collection period, by means of a syringe moistened with heparin solution. Creatinine was determined by the alkaline-picrate method[8] and PAH by a modification[9] of the method of Bratton and Marshall.[10]

Dibenamine‡ (20 mg/kg) was administered intravenously once each week for 6 weeks. The animals were divided into two groups. Group A (3 dogs) received Dibenamine in 100 ml of 0.9% NaCl solution over a period of 45 to 60 minutes. Group B (2 dogs) received the entire dose of Dibenamine in one to 5 minutes, after preliminary sedation with 15 mg/kg pentothal sodium intravenously to reduce the severity of the convulsions which

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* Aided by a grant from Givaudan-Delawanna, Inc.

† Research Fellow of the Chinese National Institute of Health. Presented in partial fulfillment of requirements for the degree of Master of Science.

[1] Hecht, H. H., and Anderson, R. B., *Am. J. Med.*. 1947, **3**, 3.

[2] Haimovici, H., and Medinets, H. E., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 163.

[3] Rockwell, F. V., *Psychosom. Med.*, 1948, **10**, 230.

[4] Nickerson, M., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1947, **89**, 167.

[5] Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

[6] Nickerson, M., and Gunn, F. D., unpublished observations.

[7] Ogden, E., *Fed. Proc.*, 1948, **7**, 87.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

[9] Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

[10] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

‡ The Dibenamine employed in these experiments was supplied by Givaudan-Delawanna, Inc., as a 5% solution in acidified alcohol-propylene glycol in sterile ampuls prepared for clinical investigation.

TABLE I.
Renal Function in Dogs Before and After Dibenamine Administration.

		Group A						Group B			
		1 (12.3 kg)		2 (13.4 kg)		3 (14.2 kg)		4 (13.6 kg)		5 (10.0 kg)	
	Dogs	G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.	G.F.R. ml/min.	R.P.F. ml/min.
	Control	46.6	130.3	61.7	181.9	56.2	168.6	57.9	163.6	42.2	121.5
	,,	45.6	125.3	60.4	178.9	57.0	166.9	57.6	160.3	41.8	122.3
	,,	45.9	126.0	60.5	180.5	58.2	172.1	57.7	162.2	41.4	121.9
	After Dibenamine										
Successive weekly inj.*	1st	46.1	110.8	58.8	162.6	56.2	165.8	58.2	150.2	42.8	144.0
	2nd	45.0	121.5	64.0	191.8	57.6	172.3	57.5	152.0	44.3	125.5
	3rd	47.6	126.5	61.7	185.2	57.0	178.4	57.2	144.2	41.0	126.4
	4th	47.6	130.0	63.2	170.0	58.8	160.4	57.7	194.5	42.4	127.8
	5th	47.8	130.6	60.1	179.2	58.8	158.2	56.9	217.2	44.4	130.9
	6th	(42.0)†	(86.2)	(62.4)	(144.6)	(56.5)	(124.7)	(57.1)	(162.9)	(44.5)	(118.2)
		47.7	127.7	60.6	188.5	56.9	155.7	57.0	200.8	44.4	132.5

* Renal function tests performed one week after each injection.

† Figures in parentheses determined at height of adrenergic blockade, 1½ hours after 6th Dibenamine injection. Note return to normal one week later.

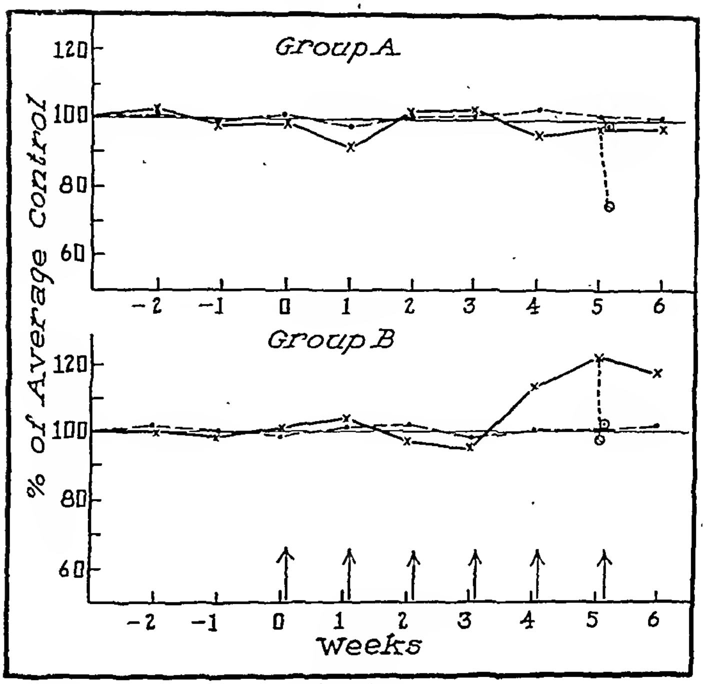

FIG. 1.

Mean glomerular filtration (•——•) and renal plasma flow (x——x) plotted as per cent of average control values for each group of dogs. Dibenamine (20 mg/kg) administered intravenously at each arrow. Circled points indicate determinations made 1½ hours after Dibenamine administration.

result from the rapid administration of such large doses of the drug. The vein was flushed with 0.9% NaCl solution at the end of the injection to prevent thrombophlebitis. Renal function was redetermined one week after *each* Dibenamine injection, and also once in each animal during the height of the Dibenamine action (1½ hours after the 6th weekly injection). Determinations of the mean systemic arterial pressure were made on several animals before and after Dibenamine administration by the use of a capillary mercury manometer and direct femoral artery puncture.

Results. The results of all clearance tests are shown in Table I and Fig. 1. In Fig. 1, mean rates of glomerular filtration and renal plasma flow are plotted as percentage of the average of three control determinations for each animal.

No persistent alterations in glomerular filtration or renal plasma flow occurred in any of the animals, with the exception of a moderate increase in renal plasma flow in one dog receiving rapid injections of Dibenamine. This increased plasma flow was accompanied by a decrease in the filtration fraction. Its significance is difficult to evaluate.

Clearance determinations made during the height of the adrenergic blocking action of Dibenamine showed a consistent reduction in renal plasma flow, which was essentially the same in the two groups; but no reduction in the glomerular filtration rate occurred. In no case did the reduction in renal plasma flow persist until the following test, *i.e.*, after wearing off of the adrenergic blocking action of the Dibenamine.

Determinations of mean systemic arterial pressure indicated about a 30% decrease 2 hours after Dibenamine administration (*e.g.*, 145 to 100 mm Hg).

With the large doses of Dibenamine employed, side-effects were not uncommon. Animals receiving the drug by slow infusion always developed hyperpnea and showed other evidence of mild central nervous system. stimulation. They salivated profusely and usually defecated. Vomiting occurred at least once in each animal, but was never observed later than the third injection in any series. As expected, the immediate toxic reactions were severe when the drug was administered in a period of 5 minutes or less. Vomiting and defecation occurred regularly and clonic convulsive movements developed after each rapid injection in spite of the barbiturate sedation. These animals were prostrated for a period of 24 to 48 hours, but appeared normal before the next weekly test. None of the animals showed any significant weight loss during the course of the experiment.

Discussion. A careful reevaluation of the renal toxicity of Dibenamine was made necessary by the work of Ogden[7] who reported that 2 weekly intravenous administrations of 20 mg/kg Dibenamine caused persistent marked reductions in renal plasma flow and glomerular filtration in one dog. He also described histological evidence of tubular degeneration. A subsequent report by the same author[11] discussed 2 additional animals given more numerous injections of Dibenamine with successively less evidence of renal damage. Ogden reported symptoms of shock in his animals immediately after the Dibenamine injections. Although injection rates were not given, it seemed possible that the alleged renal damage was related to the too rapid injection of the drug, particularly in the first animal studied. The much increased toxicity of Dibenamine after rapid intravenous injection has been repeatedly reported and warned against.[2,4,5]

Because of the possibility that a failure to observe precautions regarding the rate of Dibenamine infusion was responsible for the reported renal damage, dogs in the present experiments were divided into two groups. Group A received the drug by slow infusion with minimal side-effects. Group B received the entire dose of drug within 5 minutes for three injections, and when this procedure failed to produce persistent changes in renal function the remaining injections were completed within 1 minute each. The latter procedure provided the maximum stress possible with this dose of Dibenamine, but it failed to produce detectable renal damage.

These observations on renal function provide confirmation by more sensitive tests of the conclusion from histological observations[6] that even massive doses of Dibenamine do not produce significant renal pathology.

In the absence of a detailed report on the experiments of Ogden, we are unable to offer any explanation for the discrepancy between his results and our own. The fact that he observed decreasing renal damage in successive animals suggests that some factor in the experimental procedure may have been responsible for his findings, but our data indicate that the rate of injection is not a major factor.

The consistent decrease in renal plasma flow but not glomular filtration during the adrenergic blocking action of Dibenamine deserves special mention. This effect was observed almost equally after both slow and rapid injection. The decrease in renal plasma flow can probably be accounted for on the basis of vasodilatation in vascular beds other than renal, and the consequent lowering of the systemic arterial pressure. The decrease in blood pressure observed by direct femoral artery puncture in several animals was greater than that usually observed in anesthetized animals[4,5] or normal recumbent humans.[1,2] It is perhaps correlated with the initially high mean systemic arterial pressure in dogs and suggests that sympathetic vasomotor tone is an important factor in maintaining their blood pressure.

The increased filtration fraction (avg. 21.7%) after Dibenamine indicates a differential effect on afferent and efferent arterioles. Dibenamine has not been shown to have any direct vasconstrictor action, and the similarity of the changes observed with fast and slow injections of the drug suggests that any renal vasodilation was probably due to the adrenergic blockade *per se*. If the Dibenamine induced changes in filtration fraction are explained entirely on the basis of a release of vasomotor tone (adrenergic blockade), it must be assumed that the tonic neurogenic constriction of afferent arterioles is greater than that of efferent arterioles. This interpretation is a variance with the conclusion of Chasis, *et al.*[12] that the efferent arteriolar bed is the major site of vasomotor regulation in the human kidney, and the conclusion of Hiatt[13] that the renal circulation of the dogs has little or no vasomotor tone.

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[11] Ogden, E., Report at Meeting of Fed. of Am. Soc. for Exp. Biol., 1948.

It is clear that our present knowledge of the complex mechanism controlling renal blood flow is inadequate to provide a definitive explanation of the mechanism by which Dibenamine increases the renal filtration fraction. It is possible that in our experiments the lowered blood pressure and renal blood flow after Dibenamine induce a sufficient production of renin to alter the picture of pure vasomotor blockade. Additional experimental work is required to elucidate the mechanism of the Dibenamine induced increase in filtration fraction. Clarification of this problem may also provide a better understanding of renal vasomotor regulation.

Summary. 1. Six weekly intravenous injections of Dibenamine (20 mg/kg) failed to produce any detectable renal damage as measured by creatinine and PAH clearances in 5 trained, unanesthetized female dogs, even when the entire dose was injected within one minute so as to produce maximum toxic side-effects including prostration for 24 to 48 hours.

2. During maximal sympathetic blockade by Dibenamine, renal plasma flow was reduced 10 to 34% (average 21%), probably on the basis of reduced systemic arterial pressure. However, the filtration fraction was sufficiently elevated to provide unaltered glomerular filtration. These alterations never persisted beyond the period of active adrenergic blockade.

3. We have been completely unable to substantiate the observations of Ogden[7] on the renal toxicity of Dibenamine or to find any renal basis for his warning regarding its use. Even large doses of Dibenamine administered very rapidly appear to have no significant reno-toxic action.

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[12] Chasis, H., Ranges, H. A., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1938, **17**, 683.

[13] Hiatt, E. P., *Am. J. Physiol.*, 1942, **136**, 38.

16836

Enhancement of Penetration of Penicillin into Inflamed and Normal Mucous Membrane by Hyaluronidase.

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(Introduced by G. Shwartzman.)

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Duran-Reynals[1] first described the presence in testicular extract of a factor which is capable of facilitating the spread of vaccine virus in the skin of rabbits. Since then, this factor has been shown to be identical with hyaluronidase.[2] The enzyme has been found capable of spreading such diverse substances as vaccine virus, dyes, india ink, rabies virus, hemoglobin, diphtheria toxin, glucose and methemoglobin. It appeared to the authors that it would be of interest to determine whether penetration of penicillin into a mucous membrane might also be enhanced by hyaluronidase particularly since therapeutic failure with penicillin is sometimes ascribed to the inability of the antibiotic to reach deep-seated foci of infection. Our studies were carried out as follows:

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[1] Duran-Reynals, F., *Compt. Rend. Soc. Biol.*, 1948, **99**, 6.

[2] Chain, E., and Duthie, E. S., *Brit. J. Exp. Path.*, 1940, **21**, 324.

TABLE I.

Blood Penicillin Levels in Patients with Chronic Suppurative Sinusitis Following the Instillation of 200,000 Units of Crystalline Penicillin G into the Antrum. With and Without Hyaluronidase. (Units/ml serum.)

		Time after instillation		
Patient	Hyaluronidase	30M	60M	120M
1.	0	1.33	.8	
2.	0	.8	.57	
3.	0	1.33	.8	
4.	0	.8	.44	
5.	0	.133	.08	
6.	0	1.0	.8	
7.	0	.57	.4	
8.	0	.44	.133	
9.	0	1.33	1.0	
10.	0	1.0	.66	
11.	0	.8	.44	
12.	0	.2	.2	
13.	0	1.0	.8	
14.	0	.8	.5	
15.	0	.1	.066	
	H-1	.66	.4	
16.	0	.5	.44	
	H-1	1.33	1.0	
17.	0	.57	.44	
	H-1	1.0	.66	
18.	0	.5	.44	
	H-1	1.0	.88	
19.	0	$< .05$	$< .05$	
	H-11	.4	.133	
20.	0	.1	.2	.2
	H-11	.5	.5	.44
21.	0	$< .05$	$< .05$	
	H-11	.1	.066	
22.	0	—	.2	$< .05$
	H-11	.2	.2	.2
23.	0	.8	.57	
	H-11	1.0	.8	
24.	0	.2	.1	
	H-11	.5	.4	
25.	0	1.0	.8	
	H-11	.8	.57	
26.	0	.2	.133	
	H-11	.4	.2	

Experimental. Two groups of patients were selected for this investigation. One group consisted of 26 patients with varying degrees of chronic suppurative disease of the maxillary sinus. The other consisted of 5 subjects clinically free of sinus disease in whom antral lavage yielded a clear return.

After preliminary cocainization followed by suction and cleansing, an antral cannula was inserted into the maxillary sinus through the middle meatus. The sinus was then irrigated with physiological saline solution and the excess fluid was drained off as far as possible by tilting the head so as to make the natural

TABLE II.
Blood Penicillin Levels in Normal Subjects Following the Instillation of 200,000 Units of Crystalline Penicillin G into the Antrum. With and Without Hyaluronidase. (Units/ml serum.)

Patient	Hyaluronidase	Time after instillation		
		30M	60M	120M
A	0	.4	.08	
B	0	1.3	1.3	
C	0	.44	.2	.05
	H-11	.66	.5	.4
D	0	.5	.4	.057
	H-11	1.0	.8	.57
E	0	.5	.44	.1
	H-11	.8	.66	.3

ostia dependent. Air under 15 lb pressure was blown into the cavity in order to further empty the sinus. 200,000 units of crystalline penicillin G, dissolved in 2-3 cc of saline solution, were instilled into the sinus. Large doses were employed in order to achieve adequate blood levels so that comparisons with and without hyaluronidase would be definitive. With the crystalline preparation unlike the older amorphous penicillin local irritation was absent or minimal. The head was now tilted towards the irrigated side and kept in this position throughout the period of observation in order to prevent escape of the antibiotic. After the cannula was withdrawn a gelfoam pack was inserted into the middle meatus as an additional safeguard against leakage. In the first 3 patients tested the instilled penicillin was colored with gentian violet and upon removal of the white gelfoam pack, the absence of any stain upon it was an indication that leakage had not occurred.

Blood samples at 30, 60 and in some instances 120 minutes after the antral instillation were drawn from the antecubital vein for penicillin assay. The blood penicillin level was considered a good index of penetration since the only way in which the antibiotic could reach the blood stream was by traversing the mucous membrane. The method of assay was a tube dilution method using *Staphylococcus aureus* H as the test organism and fresh meat extract broth as the medium. The minimal concentration of standard penicillin* required to inhibit an inoculum of 5 x 10^2 *Staphylococcus aureus* H cells was 0.02 units per ml. All titrations of serum levels were compared with this standard.

The effect of hyaluronidase on penicillin penetration was investigated in a number of patients in each group by administering after a few days the same dose of penicillin with the addition of hyaluronidase. Blood samples were again assayed for penicillin and the values found with hyaluronidase and without its use were compared. Two batches of hyaluronidase† were utilized. The first, designated H-1, was employed in a dose of 20 turbidity reducing units. The second, H-11, was given in doses of 42 units since preliminary trials revealed this batch to be not as potent as H-1.

Results. The instillation of large doses of crystalline penicillin into the diseased and normal antrum was well tolerated by all the patients under study. There were no subjective complaints and no evidence of local irritative reaction following treatment. One patient proved to be allergic to penicillin and 24 hours after the instillation exhibited a diffuse skin eruption with edema of both lids. He readily responded to treatment with an antihistamine drug. On further questioning the patient recalled having experienced a similar reaction when penicillin had been administered elsewhere by the parenteral route. Thus it was found that large doses of crystalline penicillin might be safely instilled into the antral cavity provided the usual precautions in respect to allergic reactions to the drug were observed.

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* Obtained from the U. S. Department of Agriculture.

† Kindly supplied by The Schering Corporation.

As may be noted from Table I, a significant blood penicillin level was present in 24 out of 26 cases with chronic suppurative sinusitis following the intra-antral instillation of 200,000 units of the crystalline product. In every instance except one (Patient No. 25) the blood level was uniformly higher after the addition of hyaluronidase to the penicillin solution. In general, the blood levels were found to be about 2 to 3 times greater than those found without its use. Furthermore, it is of interest that patients No. 19 and No. 21 in whom no evidence of absorption could be demonstrated, showed significant levels after the addition of hyaluronidase. These findings suggest that hyaluronidase increases diffusion and penetration of penicillin through the diseased antral mucosa.

Similar findings to those noted above were obtained in subjects with non-diseased mucous membranes (Table II). A significant level was found in the blood of all 5 subjects after the antral instillation of penicillin and in each patient tested, the addition of hyaluronidase resulted in higher levels.

Although we were not primarily concerned in this study with the evaluation of the clinical effectiveness of large doses of crystalline penicillin supplemented by hyaluronidase in the treatment of chronic suppurative disease of the maxillary sinus, it is of interest that marked clinical improvement occurred in some of the patients.

Summary. The instillation of 200,000 units of crystalline penicillin G into the diseased and normal paranasal antrum is well tolerated and except for the development of an allergic reaction in one patient, was without any adverse effect. In 24 out of 26 patients with chronic suppurative disease of the sinuses and in all 5 normal subjects, a significant penicillin level in the blood was found after the intra-antral instillation.

In both groups, the addition of hyaluronidase to the instilled penicillin resulted in even higher blood levels than those found without its use, with one exception. In two patients in whom no blood level could be demonstrated, the addition of hyaluronidase resulted in a significant concentration of penicillin in the blood. It is postulated that the increased blood penicillin level following hyaluronidase is due to greater diffusion and penetration of the penicillin as a result of the spreading action of hyaluronidase.

16837

Tuberculin Reaction. III. Transfer of Systemic Tuberculin Sensitivity with Cells of Tuberculous Guinea Pigs.*

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(Introduced by E. J. Ordal.)

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In spite of many years of research by numerous investigators the passive transfer of tuberculin sensitivity was not accomplished with any degree of certainty or regularity until the important work of Chase[1] whose technic has provided a new means of exploring many aspects of this highly important type of sensitivity.†

In previous publications,[2,3] we have pre-

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* This investigation was supported by a grant from the United States Public Health Service.

[1] Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, 59, 134.

† The term sensitivity is used throughout the present article in preference to the more commonly used term hypersensitivity.

sented experimental evidence which indicates that the tuberculin reaction is the result of the interaction of a sessile antibody with specific antigen. In this work[2] the observation of Chase[1] that cutaneous tuberculin sensitivity can be passively transferred with the cells of induced peritoneal exudates of non-tuberculous donor guinea pigs sensitized with heat-killed tubercle bacilli was confirmed by the use of tuberculous donors. Another observation was that the *in vitro* effects of tuberculin in suppressing the initial migration of leukocytes from splenic explants of tuberculous guinea pigs is markedly reduced by specific desensitization.[3] In addition the results of preliminary trials were presented which indicated that homologous passive transfer of *systemic tuberculin sensitivity can be accomplished* in the guinea pig with cells of peritoneal exudates of tuberculous donors.[2]

The results given in the present report confirm and extend the observations of these preliminary trials.

Although the systemic tuberculin reaction was recognized as early as the local cutaneous reaction,[4,5] and is still used in the standardization of Old Tuberculin, it is not employed as widely as the cutaneous reaction for determining tuberculin sensitivity in experimental tuberculosis of laboratory animals. It is unfortunate that the systemic reaction commonly involves the sacrifice of these animals, since it is probably a more reliable measure of tuberculin sensitivity than the cutaneous reaction which is observed to be non-specifically affected by many factors such as age,[6,7] general health and intercurrent disease.[8-11]

Koch[4,5] observed that fatal systemic tuberculin shock in the guinea pig is so characteristic that there is little reason to confuse it with other types of shock. The principal features which Koch noted to characterize the reaction were the delayed onset, the protracted course with extreme malaise and the focal reactions at the site of tuberculous lesions.

In his studies on systemic tuberculin shock in the guinea pig Weinzirl[12] was also much impressed by the inactivity of the animals, the limpness of their muscles, and the anorexia and general prostration they exhibited. He proposed that the experimental provocation of the systemic tuberculin reaction by the intraperitoneal injection of tuberculin be called the "systemic test".

The systemic tuberculin reaction has been used in the past as an index of sensitivity in many of the numerous attempts that have been made to accomplish homologous and heterologous passive transfer of tuberculin sensitivity in laboratory animals and man.[13-23] In these attempts a variety of transfer materials were used such as serum, whole blood, exudates, transudates, and organ mashes. The results of these various experiments were irregular and inconclusive.

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[2] Kirchheimer, W. F., and Weiser, R. S., PROC. SOC. EXP. BIOL. AND MED., 1948, **66**, 166.

[3] Kirchheimer, W. F., and Weiser, R. S., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 407.

[4] Koch, R., *Dtsch. Med. Wchnschr.*, 1890, **16**, 1029.

[5] Koch, R., *ibid.*, 1891, **17**, 101.

[6] Freund, J., *J. Immunol.*, 1927, **13**, 285.

[7] Freund, J., *ibid.*, 1929, **17**, 465.

[8] Pilcher, J. D., *Am. Rev. Tuberc.*, 1930, **21**, 669.

[9] Mitchell, A. G., Wherry, W. B., Eddy, B., and Stevenson, F. E., *Am. J. Dis. Child.*, 1928, **36**, 720.

[10] Bloomfield, A. L., and Mateer, J. G., *Am. Rev. Tuberc.*, 1919, **3**, 166.

[11] v. Pirquet, C. E., *Arch. Int. Med.*, 1911, **7**, 259, 383.

[12] Weinzirl, J., *Tubercle*, 1931, **12**, 488.

[13] Friedemann, V., *Münch. Med. Wchnschr.*, 1907, **49**, 2414.

[14] Bauer, J., *ibid.*, 1909, **56**, 1218.

[15] Roepke, *Z. f. Medizinalbeamte*, 1910, No. 5, p. 149.

[16] Bail, O., *Z. f. Immunitatsforschg.*, 1909, Orig. IV, 470.

[17] Joseph, K., *Beitr. z. Klin. der Tuberk. von Bauer*, 1910, *Bd.* **17**, 461.

[18] Yamanouchi, T., *Zentralbl. f. Bakt.*, 1909, Part I, **44**, 434.

[19] Yamanouchi, T., *Comp. Rend. Soc. Biol.*, 1909, **66**, 531.

[20] Onaka, M., *Beitr. z. Klin. der Tuberk. von Bauer*, 1910, Orig. VII, 507.

[21] Kraus, R., Loewenstein, E., Volk, R., *Zentralbl. f. Bakt.*, 1911, I, **50**, 361.

[22] Massol, L., Breton, M., and Bruyant, L., *Comp. Rend. Soc. Biol.*, 1913, **74**, 185.

[23] Selter, H., *Z. f. Immunitatsforschg.*, 1921, **32**, 325.

Methods. In the present investigation the guinea pigs employed as cell donors weighed between 650 and 800 g and were sensitized 5-9 weeks prior to use by the intraperitoneal injection of approximately 5 mg of living *Mycobacterium tuberculosis* var. *bovis* of the B.C.G. strain. Only those animals showing necrotic reactions to the intradermal injection of 0.1 ml of 1:100 O.T. were used as donors. Cell exudates were induced in the donor animals as outlined by Chase[1] by the intraperitoneal injection of 25 to 30 ml of paraffin oil.‡ The animals were killed 48 hours later and the cells collected and washed by centrifugation in heparinized guinea pig serum—Tyrode solution in the manner described by Chase.[1]

The recipients were large, healthy male guinea pigs each of which was injected intraperitoneally with the washed, pooled cells from 6 donors. Each recipient was challenged 48 hours after receiving the donor cells by the intraperitoneal injection of 2 ml of O.T. (1X International Standard) diluted with 8 ml of 0.85% NaCl.§

The controls consisted of recipients given intraperitoneal injections of comparable quantities of cells from (1) normal donors, (2) donors sensitized 5 to 9 weeks previously with 5 mg of living *Mycobacterium smegmatis*, (these animals gave 3+ cutaneous reactions to smegmatis tuberculin 1:100) (3) donors injected 5 to 9 weeks previously with powdered quartz and (4) tuberculous donors desensitized by the repeated subcutaneous injection of increasing doses of O.T. in the manner previously described.[3] These controls were challenged with O.T. in the manner described above. An additional control group consisted of recipients given the cells of sensitive donors and challenged with a dose of diluted glycerine broth comparable to the concentration of these substances present in the O.T. preparation used.

The peritoneal cells of the sensitive donors and the control groups, including desensitized animals, were examined by the neutral red supravital method as outlined previously.[2] A large percentage of the cells of all of the preparations were found to be living.

Except for the development of slight abdominal tenderness in an occasional animal, all recipients appeared to remain physically normal during the 48 hour period prior to the challenging dose of shock test substance. Their rectal temperatures remained at normal levels.

Results. The results of the principal experiments are recorded in Table I. They show that of the 8 recipients of cells from sensitive animals, 5 died of tuberculin shock in from 20 to 50 hours, and 3 were severely shocked but recovered. Among the control groups one animal developed tuberculin shock.

A mild, but transient discomfort was observed in most of the animals immediately following the challenging injection of tuberculin. This was apparently due to the glycerine, since the same effect was observed following injection of normal animals with similar dilutions of glycerine and likewise in the group of recipients of the cells of sensitive animals challenged with diluted glycerine broth.

One of the recipients of cells of desensitized tuberculous donor animals developed mild symptoms of tuberculin shock and recovered. Another animal of this group showed no symptoms of shock following the challenging dose of O.T. but suddenly died at the 52nd hour. There was no indication at autopsy as to what the cause of death may have been. It seems unlikely that it was due to tuberculin shock since the characteristic symptoms of tuberculin shock were lacking.

All of the recipients of the cells of sensitive donors developed typical systemic tuberculin shock which usually began after an interval of about 3 hours and became severe by 4 to 5 hours. The first evidence of shock was the development of muscle weakness ruffled hair and increased respiration. In all instances a marked drop in body temperature occurred, which in fatal cases went as low as 34°C just prior to death. In fatal cases the severity of shock increased progressively until death and marked abdominal tenderness was observed to develop in many of the animals during the late stages of shock. In those that survived, recovery from the early symptoms of shock usually began about the 20th hour and continued gradually with the result that shock symptoms disappeared entirely by about the 48th hour.

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‡ The mineral oil used was Bayol F. distributed by Stanco Incorporated, 2 Park Avenue, New York City.

§ The Old Tuberculin used in these experiments was kindly supplied by the Lederle Laboratories of Pearl River, N. Y.

All of the animals that died were promptly autopsied. A small amount of clear yellowish and at times sanguinous fluid was commonly found in the peritoneal cavity. The omentum was rolled up and the omental folds adherent. A small amount of fibrinous deposit was occasionally present on the surface of the liver. The mesenteric vessels appeared to be engorged and a pronounced reddish discoloration of the walls of the small intestines was found with great regularity. The liver was large and distended with blood. Bacteriological examinations of the peritoneal fluid by smear and culture were negative in all instances. There were no other abnormal findings.

Summary. The results of the present investigations establish that systemic tuberculin sensitivity can be passively transferred in the guinea pig with the cells of induced peritoneal exudates of tuberculous donors. The systemic shock produced in recipients passively sensitized in this manner is of the typical delayed tuberculin type. These findings supplement the observation of Chase[1] that cutaneous tuberculin sensitivity can be passively transferred in the guinea pig with the cells of induced peritoneal exudates of donors sensitized with heat-killed tubercle bacilli by providing additional evidence that the sensitivity transferred is of the tuberculin type. In a limited number of trials it was also found that desensitization of tuberculous donors with tuberculin tends to abolish the capacity of their peritoneal cells to passively transfer tuberculin sensitivity. If this observation is confirmed it will provide strong evidence that desensitization results from the saturation of fixed cellular antibody with antigen.

TABLE I. Passive Transfer of Systemic Tuberculin Sensitivity with Cells of Induced Peritoneal Exudates of Tuberculous Guinea Pigs.

Recipient No.	Preparation of donors	Sensitizing dose of cells in ml	Dose of challenging material	Reaction of animal
1	B.C.G. sensitized	.5	10.0 ml diluted O.T.	Died at 30 hrs.
2	,, ,,	.5	,, ,, ,, ,,	,, ,, 21 ,,
3	,, ,,	1.0	,, ,, ,, ,,	,, ,, 21 ,,
4	,, ,,	.5	,, ,, ,, ,,	In severe shock at 4 hrs, fully recovered at 30 hrs.
5	,, ,,	.5	,, ,, ,, ,,	Died at 20 hrs.
6	,, ,,	.5	,, ,, ,, ,,	Severe shock at 4 hrs, fully recovered after 48 hrs.
7	,, ,,	.8	,, ,, ,, ,,	Severe shock at 5 hrs, fully recovered after 48 hrs.
8	,, ,,	.5	,, ,, ,, ,,	Died at 50 hrs.
Controls				
1	*M. smegmatis* sensitized	.5	,, ,, ,, ,,	No symptoms.
2	B.C.G. sensitized and desensitized	1.8	,, ,, ,, ,,	Slight symptoms shock 4 hr, fully recovered 10 hr
3	,, ,, ,, ,,	.6	,, ,, ,, ,,	No symptoms.
4	,, ,, ,, ,,	.7	,, ,, ,, ,,	No symptoms, but died suddenly after 52 hrs.
5	Quartz treated	.5	,, ,, ,, ,,	No symptoms
6	,, ,,	.8	,, ,, ,, ,,	,, ,,
7	Normal	.5	,, ,, ,, ,,	,, ,,
8	,,	1.2	,, ,, ,, ,,	,, ,,
9	B.C.G. sensitized	.9	10.0 ml diluted glycerine broth	,, ,,
10	,, ,,	.5	,, ,, ,, ,, ,,	,, ,,

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Carious Lesions in Cotton Rat Molars. II. Effect of Removal of Principal Salivary Glands.*

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An appreciable increase in susceptibility to dental caries has been observed in albino rats which were maintained on coarse particle diets after the removal of some or all of the principal salivary glands.[1-4] Similarly, a substantial increase in susceptibility to tooth decay has been observed in Syrian hamsters after the extirpation of the major salivary glands.[5] The diets used in these investigations were composed primarily of natural foodstuffs. In the present experiments, the principal salivary glands of weanling cotton rats were removed prior to periods of maintenance on caries-producing, purified rations to determine if this procedure would result in a greater number and extent of carious lesions and a different distribution of the lesions with respect to the various tooth surfaces.

Experimental. The cotton rats to be used in this experiment were separated from their mothers at 14 days of age and divided into two groups, equal with respect to parentage, sex and weight. The individuals in the one group were kept as intact controls, while the principal salivary glands of the cotton rats in the other group were removed under ether anesthesia. After the operation, each animal was placed in an individual wire-bottom cage with access to water and to either of 2 caries-producing purified rations *ad libitum.* Half of the cotton rats in each of the experimental and control groups were provided with ration 100.[6] The basal composition of this ration was: sucrose 67%, casein 24%, salt mixture 4% and corn oil 5%, supplemented with adequate amounts of the fat-soluble vitamins, the B vitamins and liver concentrates to permit normal growth. The other half of the cotton rats in each of the intact and operated groups was fed a caries-producing ration of slightly lower caries-producing properties, ration 130, which was a slight variant of ration 100 attained through the isocaloric replacement of 18 of each 67 parts of sucrose by 8 parts of lard by weight. When rations comparable to ration 130 were fed after the development of the teeth was largely complete, the carbohydrate-protein-fat distribution has been shown previously to be borderline in its ability to reduce the susceptibility of the molar teeth of cotton rats to the initiation and development of carious lesions during a 14 week experimental period.[7-9] At the end of 12 weeks after desalivation, all cotton rats were sacrificed and the number and extent of the carious lesions determined for each animal by previously described criteria.[10,11]

Results. The average weight gain during

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* This project was supported in part by a grant from the Sugar Research Foundation, Inc., New York. We are indebted to Merck and Co., Rahway, N. J., for gifts of the B-complex vitamins used in this study.

[1] Kondo, S., Ichikawa, T., and Arai, M., *Tr. Soc. path. jap.*, 1938, **28**, 461.

[2] Cheyne, V. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 587.

[3] Weisberger, D., Nelson, C. T., and Boyle, P. E., *Am. J. Orthodontics and Oral Surg.*, 1940, **26**, 88.

[4] Hukusima, M., *Tr. Soc. path. jap.*, 1940, **30**, 245.

[5] Gilda, J. E., and Keyes, P. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 28.

[6] Shaw, J. H., *J. Dental Research*, 1947, **26**, 47.

[7] Schweigert, B. S., Shaw, J. H., Zepplin, M., and Elvehjem, C. A., *J. Nutrition*, 1946, **31**, 439.

[8] Schweigert, B. S., Potts, E., Shaw, J. H., Zepplin, M., and Phillips, P. H., *J. Nutrition*, 1946, **32**, 405.

[9] Shaw, J. H., unpublished data.

[10] Shaw, J. H., Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Phillips, P. H., *J. Nutrition*, 1944, **28**, 333.

[11] Shaw, J. H., Schweigert, B. S., Elvehjem, C. A., and Phillips, P. H., *J. Dental Research*, 1944, **23**, 417.

TABLE I.
Effect of Extirpation of Principal Salivary Glands on Rate of Growth and Incidence and Extent of Carious Lesions in the Cotton Rat.

Ration	Condition	No. of rats	Avg gain in g	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	Normal	7	50	22.1	2.0		50+	8+	
						5.1			5.6
”	“Desalivated”	9	28	33.9	1.1		109+	7+	
130	Normal	4	54	17.0	2.1		31+	9+	
						3.1			3.0
“	“Desalivated”	5	42	25.0	1.6		63+	6+	

* S.E.M., Standard error of means.
† C.R., Critical ratio.

the experimental period and the average number and extent of carious lesions developed in intact cotton rats and littermates from which the major salivary glands were removed are presented in Table I. Those cotton rats which had had their principal salivary glands removed prior to 12 weeks maintenance on ration 100 had a 53% higher average number of carious lesions and a 110% higher average extent of carious lesions than their intact littermates which had been fed ration 100 for the same period. Likewise, the desalivated animals which had been maintained on ration 130 had a 47% higher average number of carious lesions and a 100% increase in the average extent of carious lesions over that demonstrated by their normal littermates which had received ration 130. These increases in average number and average extent of carious lesions were sufficiently large to be judged statistically significant for both rations. As has been previously demonstrated, the intact cotton rats which were maintained on ration 130 for the 12 week experimental period had a slightly lower average number and average extent of carious lesions than their normal littermates which were maintained on ration 100. Likewise, the desalivated cotton rats which were maintained on ration 130 had an appreciably lower average number and average extent of carious lesions than their desalivated littermates which were maintained on ration 100 throughout the experimental period. Thus the post-developmental caries-retarding effect of ration 130 occurred not only in normal but in desalivated cotton rats and to approximately the same degree in both the intact and operated animals.

Despite the fact that the average number and the average extent of the carious lesions were significantly higher in desalivated cotton rats than in their intact littermates, almost all carious lesions in intact and operated animals developed in the sulci of the molars except for a few lesions on the proximal surfaces of the molars. The increased occurrence of carious lesions in desalivated cotton rats was a result of the involvement of a higher percentage of the sulci and of the proximal surfaces than occurred in normal animals. No carious lesions were found on the smooth surfaces nor on the occlusal surfaces in either intact or desalivated animals.

At the end of the 12 week experimental period, the crowns of the molars in all 4 quadrants of the desalivated cotton rats which had received ration 100 had been considerably reduced in volume by tthe carious processes and the subsequent fracture of undercut cusps. The remaining areas of the smooth surfaces were heavily covered with food debris but no gross lesions were observable under this material by the methods of detection used. In the cotton rats fed ration 130 much less of the molar crowns had been destroyed by the carious processes; thus the size of the smooth surfaces had not been reduced as much but were as heavily covered with food debris. Again no carious lesions were observed on the smooth surfaces. Thus the relative distribution of carious lesions on the various tooth surfaces in the desalivated cotton rat appeared to be identical with that in the normal cotton rat.

The rate of growth was approximately the same for normal cotton rats receiving rations 100 and 130. The rate of growth for the desalivated cotton rats was appreciably less than for their intact littermates. This reduction in rate of growth was greatest in the animals which received ration 100 throughout the experimental period.

Summary. The removal of the principal salivary glands of cotton rats prior to maintenance on purified, caries-producing rations resulted in a significantly greater average number and average extent of carious lesions than was observed in their intact littermates maintained under otherwise identical experimental circumstances. No alteration in the relative distribution of the lesions with respect to the various tooth surfaces resulted from the removal of the major salivary glands.

16839 P

Inhibition of the Appearance of Phenol Red in Frog Kidney Tubules *in vitro.*

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4′-Carboxyphenylmethanesulfonanilide (caronamide) inhibits concentration of phenol red in the lumina of the tubules of frog kidney slices.† This observation suggested that it might be of interest to study the activity of various other substances as inhibitory agents. A number of compounds have been studied. These have been mainly carboxylic acids; however, the present communication is concerned only with derivatives of cinchoninic acid. A recent study in this laboratory has revealed that certain derivatives of cinchoninic acid inhibit a water diuresis in the dog.[1] Some of these compounds have been found to increase uric acid excretion in man.[2-4] The experiments described in this report show that the appearance of phenol red color in the lumina of the tubules in frog kidney slices is inhibited by certain of these substances.‡

Procedure. A modification of the method of Forster has been used.[5] Both kidneys of a frog (*Rana pipiens*) were removed and sliced transversely into sections approximately 200 microns in thickness. The slices were mixed and distributed to 12 small Petri dishes each containing 5 ml of the solution described by Forster.[5] The concentration of phenol red used was 0.03 millimoles per liter. The substances being tested were dissolved in this

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* Parke, Davis Fellow in Pharmacology and Experimental Therapeutics.

† Dr. E. K. Marshall, Jr., in conjunction with Dr. Roy P. Forster, made this observation on the fish kidney (flounder) in Maine in the summer of 1947.

[1] Marshall, E. K., Jr., and Blanchard, K. C., *J. Pharm. and Exp. Therap.*, in press.

[2] Nicolaier, A., and Dohrn, M., *Deutsches Arch. f. klin. Med.*, 1908, **93**, 331.

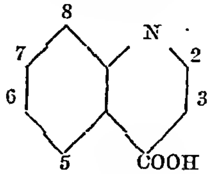
[3] Berlingozzi, S., and Donatelli, G., *Boll. Chim. Farm.*, 1936, **75**, 387.

[4] Impens, E., *Arch. de Pharmacodynamic*, 1911-12, **21-22**, 379.

‡ We wish to thank Dr. K. C. Blanchard for supplying the cinchoninic acid derivatives.

[5] Forster, Roy P., *Science*, 1948, **108**, 65.

TABLE I.
Effect of Cinchoninic Acid Derivatives on the Concentration of Phenol Red by Frog Kidney Tubules *in Vitro*.



	Min. inhibitory conc.		
Substituent	Cinchoninic derivative mM/1	Caronamide mM/1	Ratio*
None	2	.5	.25
2-Hydroxy	>2	.25	< .12
2-Hydroxy-3-methyl	>2	.3	< .15
2-Hydroxy-3-phenyl	4	.25	.06
2-Methyl	1	.125	.12
3-Hydroxy-2-methyl	0.1	.3	3
3-Carboxy-2-methyl	>4	.125	< .03
2-Phenyl	.125	.125	1
3-Hydroxy-2-phenyl	.03	.125	4
6-Methoxy	.08	.15	2

* The ratio of the minimal inhibitory concentration of caronamide to the minimal inhibitory concentration of the unknown.

solution before the kidney slices were placed therein. Oxygen was continuously bubbled through the solution in each dish by a small jet made from a 27 gauge hypodermic needle. These 12 jets were fed from a manifold.

Caronamide was used as a standard of comparison. Each experiment included three concentrations of caronamide and two controls containing no drug. In all cases the concentrations of the substances being tested were varied by a factor of two. To avoid subjective errors the minimum concentration of drug which completely prevented the appearance of phenol red color in the tubule lumina was used to evaluate effectiveness. Decrements in drug concentration below the minimal inhibitory level resulted in graded increments in the amount of color seen in the tubule lumina. The minimal inhibitory concentration of caronamide varied somewhat from one experiment to another.

Results and discussion. In Table I the inhibitory concentrations of various substances are compared with the concentration of caronamide necessary to produce the same effect in the same experiment. Only the 3-hydroxy-2-methyl, the 3-hydroxy-2-phenyl and the 6-methoxycinchoninic acids were more active than caronamide, while the 2-phenyl derivative (cinchophen) was equally active.

The desirability of comparing these results with results obtained *in vivo* in mammals is obvious. We have therefore begun such a study. In preliminary experiments, it appears that some members of this group depress the clearance of phenol red in the dog.

Summary. Cinchoninic acid and nine of its derivatives have been studied with respect to the inhibition of the appearance of phenol red color in the lumina of the tubules of frog kidney slices *in vitro*. Three of these have been found more active in this respect than is caronamide and one equally active.

16840 P

Subcortical Centers as Pacemakers of Cortical Activity.*

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In continuation of a previous investigation[1] in which the effect of anoxia and asphyxia on normal and convulsive potentials of the cortex was studied, experiments were undertaken to determine the hypothalamic-cortical relations under these conditions. All experiments were performed on curarized "Dial" cats (intocostrin, artificial respiration) in which after exposure of the brain cortical, hypothalamic, and thalamic potentials were recorded with mono- and bipolar silver electrodes. An Offner inkwriter and amplifier were used. Subcortical electrodes were inserted with the Horsley-Clarke apparatus. Strychnine pledgets served to elicit local cortical spikes; this drug was also injected either into the hypothalamus or intravenously.

In experiments in which the cortex was strychninized the results were similar to those discussed in the preceding paper. However, if strychnine was injected intravenously a series of very regular small spikes appeared synchronously in cortex and hypothalamus at a time when due to the asphyxia normal and convulsive potentials had either completely disappeared or were greatly reduced in amplitude and frequency. This burst of activity lasted in the majority of observations for 16 to 25 seconds although in some instances it was as brief as 6 or as long as 34 seconds. The frequency of discharge was variable. In general the amplitude declined greatly as the frequency increased, the maximum being about 14 to 18 per second.

Since the synchrony of discharge suggested a subcortical pacemaker strychnine was injected into the hypothalamus and in other instances into several nuclei of the thalamus. Although heavy strychninization of the hypothalamus may lead to appearance of spikes in the cortex[2] injection of this drug into a minute area of the hypothalamus induced local spiking but did not interfere with normal cortical activity and the asynchrony existing between different cortical areas. Asphyxia caused under these conditions likewise the appearance of synchronous spikes in various cortical areas and in the hypothalamus (Fig. 1). These potentials were larger in monopolar than in bipolar recordings. Cathode ray oscillograms showed that they consisted of bipolar spikes. This phenomenon was reversible and could be repeated many times in the same preparation. As time progressed the spikes appearing in the injected area became less in frequency and amplitude and then disappeared. Even at this stage the synchronous discharge could be induced in asphyxia. When some time later synchrony was no longer elicited it could be reinvoked after reinjection of strychnine into the hypothalamus.

The synchronous discharges discussed in this paper were recorded bilaterally in the cortex after unilateral injection of the hypothalamus or thalamus with strychnine. They were likewise found in contralateral thalamic nuclei. This phenomenon occurred in asphyxia as well as in anoxia (inhalation of nitrogen or nitrogen-air mixtures). It is probably related to the synchronous spindles which van Harreveld[3] observed in the cortex after prolonged asphyxia. It is suggested that:

1. Hypothalamic and closely related thala-

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* Aided by a grant of the Office of Naval Research.

[1] Gellhorn, E., and Heymans, C., *J. Neurophysiol.*, 1948, **11**, 261.

[2] Murphy, J. P., and Gellhorn, E., *J. Neurophysiol.*, 1945, **8**, 341, 431.

[3] van Harreveld, A., *J. Neurophysiol.*, 1947, **5**, 361.

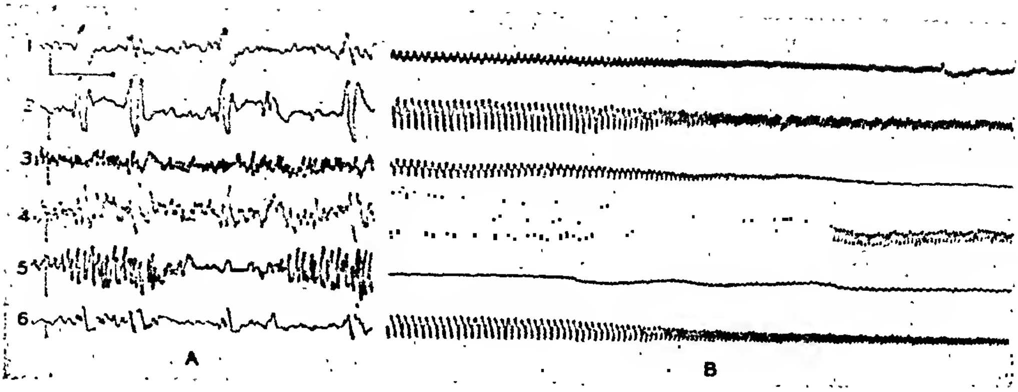

FIG. 1.

Dial cat. Right hypothalamus injected with 0.03 mg strychnine. A, control; B, during asphyxia, immediately after normal and convulsive potentials had been abolished. Note asynchrony in control and synchronous discharge in asphyxia.

1. Left ventrolateral thalamus (monopolar).
2. Right hypothalamus (monopolar) injected with strychnine.
3. Left motor cortex (bipolar).
4. Same site, monopolar.
5. Left auditory cortex (bipolar).
6. Same site, monopolar.

Calibration 100 microvolts and 1 second.

mic nuclei[2,4] do not act as pacemakers of cortical activity as long as the excitability of the cortex is unchanged.

2. In conditions such as asphyxia and anoxia which lead to a relative predominance of hypothalamic excitability the discharge of these subcortical nuclei determines the qualitative and quantitative activity in thalamus and cortex and leads to periods of complete synchrony of hypothalamic, thalamic and cortical activity.

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[4] Gellhorn, E., and Ballin, H. M., *Am. J. Physiol.*, 1946, **146**, 630.

16841

Relation of Iodinated β-amylose (Tridine) to Some Thyroid Functions.

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The appearance of iodinated β-amylose, "Tridine",† a compound of carbohydrate and iodine, suggested a biological assay to determine whether it behaves like an inorganic salt of iodine or like other iodinated organic compounds such as thyroxin or thyroglobulin.

Methods. White albino rats were used throughout the experiment. All animals received ground dog biscuit‡ and water *ad libitum* for 5 to 7 days before any experiment was begun. During the experimental period the control group remained on this diet while

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† According to the manufacturer, The Clairmint Chemical Co., 7 Lackawanna Avenue, Newark 2, N. J. "Tridine" is iodinated β-amylose and contains approximately 20% of iodine by weight. The Clairmint Chemical Co., defrayed part of the expense of this investigation.

‡ Purina Laboratory Chow.

TABLE I.
Effects of Tridine and Thyroxine on Thiouracil-induced Hypothyroidism.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	10 days
Group 1 (6)† Control	5.4 ±0.37	9.0 ±0.73	30.3 ±1.22	0.68 ±0.019	4.0 ±0.16	77.0	81.0 + 4.2%
Group 2 (6) 0.1% Thiouracil	6.3 ±0.33	15.9 ±0.51	26.0 ±1.14	0.75 ±0.023	5.2 ±0.25	82.6	67.0 —18.9%
Group 3 (6) 0.1% Thiouracil + 3% Tridine	6.5 ±0.36	13.2 ±0.73	26.5 ±0.73	0.77 ±0.080	4.1 ±0.26	76.8	64.2 —16.4%
Group 4 (6) 0.1% Thiouracil + Thyroxine	6.2 ±0.15	9.9 ±0.66	28.1 ±1.77	—0.74 ±0.016	3.7 ±0.14	—	79.3

* Calories/24 hr/kg$^{3/4}$.
† No. of animals.

the other groups received their medication in addition as a dietary supplement. The food intake was recorded and the animals were weighed every other day. The animal room temperature was maintained at 27° ±1°C.

At the beginning and termination of each experimental period the resting, fasting (24 hr) metabolic rates were determined by the use of a modified Haldane apparatus.[1] By the use of the preliminary fast and an alternate carbon dioxide absorbing jar which was switched on only when the rats were inactive it was possible to approximate the basal metabolic rate. The metabolic rates were calculated as calories per 24 hours per kilogram to the three-fourths power.

At the end of each experimental period, the animals were sacrificed and the pituitary, thyroids, adrenals, liver, and kidneys weighed and computed as weight of organ per hundred grams of body weight. Histological sections were taken when indicated and stained with hematoxylin and eosin.

Results. The mean results with their standard errors are given in the tables. Any change that could occur by chance in only 5% or less of the trials is considered significant.

Experiment I. Twenty-four adult (150 g) female rats were used in this series. After a preliminary period of 7 days, the metabolic rates were determined and medication begun. For this purpose they were divided into 4 groups of 6 animals each. Group 1 served as controls, Group 2 received 0.1% thiouracil in the food, Group 3 received 3% tridine and 0.1% thiouracil in the food, and Group 4 received 0.1% thiouracil in the food and 0.02 mg of *l*-thyroxine injected subcutaneously every other day. After 10 days a final metabolic rate determination was made and the animals sacrificed, the organs weighed, and histological sections taken of the thyroid and kidney of all animals. The data are assembled in Table I. Histologically, all the animals showed normal renal tissue. Group 1 showed normal thyroid tissue. Group 2 showed changes in the thyroid typical of thiouracil medication, namely, nearly complete disappearance of the colloid substance together with marked hyperplasia of the epithelial cells with a change from the cuboidal to the high columnar form. In Group 3 this picture was modified so that colloid was present and more abundant than in the previous group, but it was still subnormal in amount. There was also less evidence of epithelial hyperplasia, but it was present to a slight degree. Group 4 showed thyroid sections which were identical with the control group.

Tridine had no significant effect on the lowered metabolic rate of thiouracil-treated

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[1] Anderson, J. T., and Nasset, E. S., *J. Nutrition*, 1948, **36**, 703.

TABLE II.
Effect of Tridine on Thyroxine-treated Adult Rats.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	10 days
Group 5 (6)† Thyroxine	7.7 ±0.54	9.1 ±0.51	29.6 ±1.23	0.82 ± .014	4.2 ±0.11	79.8	91.5 +14.7%
Group 6 (6) Thyrox. + 1% Tridine	7.1 ±0.42	9.3 ±0.78	31.5 ±1.67	0.94 ±.030	4.5 ±0.11	79.0	90.5 +14.6%
Group 7 (6) Thyrox. + 3% Tridine	6.7 ±0.42	10.8 ±1.61	30.1 ±3.39	0.92 ±0.040	4.5 ±0.28	76.5	83.8 + 9.5%
Group 8 (6) Thyrox. + 5% Tridine	7.5 ±0.19	10.8 ±0.30	32.8 ±3.65	0.95 ±0.074	5.3 ±0.66	77.0	86.1 +11.8%

* Calories/24 hr/kg¾.
† No. of animals.
All rats received 0.02 mg thyroxine subcutaneously every other day.

rats. (The standard error of the metabolic rate determination is about 2.5% of the total value). The thyroid glands of Group 3 were significantly smaller than those in Group 2 (thiouracil alone) but it will be shown later that this is not a specific effect of tridine.

Experiment II. Twenty-four adult (190 g) female rats were divided into 4 groups of 6 rats each and after an acclimatization period of 5 days, metabolic rates were determined and medication begun. All received 0.02 mg of thyroxine subcutaneously every other day. Group 5 received stock diet alone, while Group 6 received 1% tridine, Group 7 received 3% tridine, and Group 8 received 5% tridine, all mixed with the stock diet. After 10 days another metabolic rate determination was made, the animals sacrificed, and organ weights recorded. No histological sections were taken. Results are set out in Table II.

This experiment was designed to test the possible effect of feeding tridine (1, 3 and 5% of the diet) on hyperthyroidism produced by injection of thyroxine. The only significant change in all of these observations was an increase in the size of the thyroids of Group 8. All of the other organs as well as the energy metabolism were not significantly altered by tridine treatment. It is evident, therefore, that tridine in the dosages employed does not exhibit any marked antagonism to thyroxin.

Experiment III. It was of some interest to determine what influence the feeding of tridine might have on the growth of young animals. Twenty rats of both sexes about 4 weeks old were divided into 4 groups of 5 rats each and after a preliminary period of 5 days, the metabolic rates were determined and the animals started on the medication. Group 9 received stock diet, Group 10 received 1% tridine, Group II received 3% tridine and Group 12 received 5% tridine, all mixed with stock diet. After 18 days, the metabolic rates were again determined, the animals sacrificed, organ weights recorded, and histological sections of the thyroid gland taken. Table III shows the organ weights and the metabolic rates. Table IV shows weight gains, food consumption and efficiency of food utilization. There were no fatalities in any of the groups.

Histologically, the thyroid glands showed little deviation from the normal. There was slightly more colloid material present in those rats receiving 3% and 5% tridine than in the control and the 1% tridine group. There was, however, no evidence of epithelial hyperplasia. It is evident from Table III that the thyroid in Group 10 was significantly larger than the control (Group 9). The adrenal glands and the liver were significant-

TABLE III.
Tridine as a Dietary Supplement for Young Rats.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	18 days
Group 9 (5)† Control	4.7 ±0.11	8.5 ±0.81	20.0 ±0.50	0.93 ±.028	6.2 ±0.11	98.0	88.6 — 9.6%
Group 10 (5) 1% Tridine	5.1 ±0.49	12.3 ±0.67	22.2 ±0.69	0.94 ± .032	6.7 ±0.19	99.5	88.1 —11.5%
Group 11 (5) 3% Tridine	4.6 ±0.24	9.9 ±0.96	23.8 ±0.47	0.97 ±.021	7.3 ±0.23	100.0	89.9 —10.1%
Group 12 (5) 5% Tridine	5.6 ±0.71	10.0 ±1.58	29.4 ±4.75	1.05 ±.104	6.7 ±0.71	99.0	80.3 —19.0%

* Calories/24 hr/kg¾.
† No. of animals.

TABLE IV.
Food Utilization in Young Rats as Affected by Tridine Supplement in the Diet.

	Avg wt				
	0 days g	16 days g	Avg gain g	Avg food intake g	G food eaten per g wt gained
Group 9 Control	56.0	136.4	80.4	200.9	2.5
Group 10 1% Tridine	53.6	118.4	64.8	172.4	2.7
Group 11 3% Tridine	50.8	104.6	53.8	147.6	2.7
Group 12 5% Tridine	50.8	76.4	25.6	112.9	4.5

ly larger in Groups 10 and 11. The energy metabolism was depressed significantly in Group 12 as compared with the other groups which exhibited only the normal decline in metabolic rate associated with maturation. Table IV shows that with all dosages of tridine there was a loss of appetite and a corresponding failure to gain weight. In Group 12 weight gained per gram of food eaten was very low and taken together with the depressed metabolism may be regarded as a sign of toxicity with possible impaired digestion and absorption of food. There was slight but inconclusive histological evidence of increased colloid production induced by tridine. There was, however, no increase in the metabolic rate to corroborate this finding. The maximum tridine intake was approximately 5.5 g per kilo per day.

Experiment IV. Ten young (125 g) rats were used, 4 males and 6 females, equally divided into the 2 groups of 5. After a preliminary period of 5 days, metabolic rates were determined and medication was begun. Both groups received 0.1% thiouracil in the stock diet and in addition Group 13 received 3% tridine and Group 14 received 0.77% potassium iodide in the diet. These percentages represent equivalent amounts of iodine. After ten days a final metabolic rate was determined, the animals sacrificed, their organs weighed, and histological sections made of the thyroid glands. Results are recorded in Table V. Histological sections showed a modified thiouracil effect in both cases, the picture being similar to that of Group 3 in Experiment 1. In both groups there was a partial reversal of the thiouracil effect. There are no signifi-

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Lesions of the Islands of Langerhans Produced by a Styryl Quinoline Derivative.

F. D. W. Lukens and W. B. Kennedy.

From the George S. Cox Medical Research Institute, University of Pennsylvania.

Dunn, Sheehan and McLetchie[1] first reported experimental necrosis of the islands of Langerhans. Although most of this report concerns the lesions produced by alloxan, they refer to some experiments in which styryl quinoline No. 90 caused injury of the islands. As the papers cited in this article contained no information concerning the effects on the pancreas, the only report of pancreatic lesions is that observed by them.[1] Since this report, Goldner and Gomori[2] have studied quinoline, cincophen and quinone. These were without effect on the pancreas. Kennedy and Lukens (unpublished) found sublethal doses of quinoline ineffective.

Because of the pancreatic damage ascribed to styryl quinoline, we have examined certain quinoline compounds with respect to their possible pancreatotoxic effect.

Methods. Normal male rabbits were kept in metabolism cages and fed an unmeasured diet of cabbage and oats. Each compound was given (a) intravenously and the response observed for 2-5 days; (b) subcutaneously every day for 2 weeks and (c) in 2 animals one compound was given by stomach tube daily for 2 weeks. The urine was tested daily for sugar, acetone, albumin and specific gravity. Occasional microscopic examinations of the urine sediments were made. The blood glucose was determined once or more on most animals. In taking the blood from the ear, the animals were not trained to this procedure, a fact which presumably accounts for the slightly elevated values obtained. Except for rabbits dying acutely from the drugs or from incidental illnesses, all of them looked well, ate well and maintained their weight. At the end of the experiments they were given nembutal and autopsied. In all cases, the pancreas, liver and kidneys were taken for microscopic examination. In a few animals other organs were sectioned also.

Two compounds were also tested in rats by subcutaneous administration.

The substances tested were as follows:

I 7-chloro-4-(4-diethylamino-1-methylbutylamino)-3-methyl*quinoline*, bis-(acid sulfate), hemihydrate.

II 7-chloro-4-(4-diethylamino-1-methylbutylamino)*quinoline*, diphosphate.

III 1-(7-chloro-4-quinolylamino)-3-diethylamino-2-*propanol*, diphosphate.

IV 4-(7-chloro-4-quinolylamino)-α-diethylamino-*o-cresol.*

V 2-[2-(1-amyl-2, 5-dimethyl-3-pyrryl) vinyl] - 1,6-dimethyl-quinoliniumchloride.

VI Styryl Quinoline: 2 (p-acetylaminostyryl) 6-dimethylaminoquinoline-methochloride.

VII N-[2-(p-Diethylaminostyryl)-6-methylquinoline] ethochloride.

Results. In the case of compounds I to VI, there was neither physiological nor histological evidence of pancreatic damage. The results have been summarized in Table I. Animals dying in a few minutes have been excluded from this summary.

The kidneys deserve particular mention because of Sheehan's[1] report of nephritis from styryl quinoline. No gross injury was found in any experiments. The slight clouding on the tests for albumin never exceeded that seen in numerous normal rabbit urines. The specific gravity varied widely and included samples with good concentration, all resembling normal rabbits on the same diet.

In addition to the absence of diabetes, there was no evidence at autopsy of any harmful

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[2] Goldner, M. G., and Gomori, G., *Endocrinology*, 1944, **35**, 24.

TABLE V.
Comparison of Equivalent Amounts of Tridine and KI in Thiouracil-treated Rats.

	Organ wt per 100 g body wt					Metabolic rate*	
	Pituitary mg	Thyroid mg	Adrenals mg	Kidneys g	Liver g	0 days	10 days
Group 13 (5)†	5.4 ±0.32	13.7 ±1.13	18.8 ±2.55	0.99 ± .062	5.7 ±0.33	86.3	73.5 —14.8%
Group 14 (5)	5.7 ±0.73	12.5 ±1.78	18.9 ±2.57	0.86 ± .032	4.9 ±0.26	79.6	72.4 —10.0%

Group 13—0.1% Thiouracil in diet + 3% Tridine.
Group 14—0.1% Thiouracil in diet + 0.77% KI (≎ 3% Tridine in I).
* Calories/24 hr/kg¾.
† No. of animals.

cant differences between these groups in organ weights, energy metabolism or histological appearance of the thyroid gland. It is concluded that tridine exerts no unique effect which cannot be accounted for on the basis of its iodine content.

Discussion. It is evident from the results that tridine, in the animal body, behaves like an equivalent amount of inorganic iodine. Like KI,[2] tridine is capable of preventing partially the thyroid hyperplasia which accompanies thiouracil feeding. The low metabolic rate of thiouracil-treated rats is unaffected by feeding tridine as 3% of the diet. Moderate hyperthyroidism produced by the administration of thyroxin does not seem to be influenced significantly by the addition of tridine as a dietary supplement in concentrations of 1, 3 and 5%. From the fact that tridine is approximately equivalent, on the basis of iodine content, to KI in thiouracil-treated rats it may be concluded that the new compound is readily absorbed but it is not known whether it is hydrolyzed before absorption. Since starch is so readily digested it seems likely that β-amylose, a derivative of starch, should also be split to simpler units. It would be interesting to know whether ionic iodine is released in the lumen of the small intestine.

The deleterious effect of feeding tridine to growing rats is evidently closely related to an impaired appetite. The ingestion of tridine prevented maximal gains in weight. When the diet contained 5% of tridine the gain in weight was less than one-third of the control value and the food was very poorly utilized. It would be interesting to know whether this was due to non-absorption or to excessive waste after the foodstuffs had reached the blood stream.

Summary. 1. Iodinated β-amylose, tridine, prevents partially the thyroid hyperplasia caused by thiouracil. In this respect it acts like potassium iodide. Tridine, except in toxic concentrations has no effect on the metabolic rate.

2. In rats receiving thyroxin, tridine, in dosages of 1, 3 and 5% of the diet, has no significant effect on either the size of the organs or the metabolic rate.

3. In young rats all dosages of tridine (1, 3 and 5%) are deleterious with respect to consumption of food and gain in body weight.

4. In thiouracil-treated rats 3% tridine is indistinguishable in its effects from an equivalent (0.77%) amount of KI.

5. The data from the present series of experiments on 78 rats suggest that tridine when administered by mouth exerts its effect by virtue of its iodine content and when given in equivalent amounts cannot be distinguished from KI.

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[2] Sadhu, D. P., *Am. J. Physiol.*, 1948, 152, 150.

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TABLE I.
Absence of Diabetogenic Effect of 6 Quinoline Compounds.

Compound (see text)	No. rabbits	Dosage		Blood glucose		Days until autopsy (range) days
		Amt mg/kg	Route*	No.	Range mg/100 ml	
I	8	10–20	i.v.	10	102-144	3–8
	2	20	oral	6	109-131	14–15
	2	20	s.c.	7	115-153	12–15
II	6	20	i.v.	6	103-121	4–5
	5	20–40	s.c.	12	102-130	15
III	6	20	i.v.	5	105-128	4–5
	4	20	s.c.	8	108-125	14
IV	6	10–20	i.v.	6	107-124	4–6
	6	20	s.c.	14	106-151	11–15
V	2	2–4	i.v.	0		1
	9 rats	4–16	s.c.	5	125-162	4–5
VI	4	10–30	s.c.	0		2

* i.v. = intravenous; s.c. = subcutaneously.

TABLE II.
Effect on Pancreatice Islets of Compound VII: N[2(p-Diethylaminostyryl)-6-methyl quinoline] ethochloride.

Animal and No.	No. in group	Doses × days, mg/kg	Time of autopsy, days	Necrosis of islets	Blood sugar at autopsy
		A. Lesions observed.			
Rabbit 70		15 × 1 i.v.*	<1	+++	
" 76		30 × 1 i.v.	<1	+	
Rat 1		150 × 1 s.c.	1	+	
" 3		60 × 1 s.c.	1	+	
" 5		10 × 1 s.c.	1	+	
" 6		10 × 1 s.c.	1	+	
" 10		5 × 1 s.c.	1	+	
" 17		2 × 2 s.c.	1	+	
		B. No lesions present.			
Rabbits	8	10-30 × 1 i.v.	<1 to 5	0	121-178 (5 rabbits)
Rats	12	10 × 1 to 1 × 6 s.c.	1 to 6	0	101-702 (7 rats)

* i.v. = intravenously; s.c. = subcutaneously.

effect on the liver or other organs studied. The lungs, thyroid, thymus, adrenals, spleen were all grossly normal. Microscopic examination showed that the pancreas and islands of Langerhans were entirely normal. The liver, kidney and other organs showed no definite abnormality.

Of 28 animals tested with compound VII, 8 showed evidence of early necrosis of the islands of Langerhans. Table II summarizes these experiments, and Figures 1-4 illustrate the lesions in 2 of these animals. A normal island and the early necrosis from alloxan are presented for comparison. Early necrosis is shown by the shrunken, pyknotic nuclei and by the loss of normal granulation of the cytoplasm. There was no consistent relation of these lesions to dosage; they seemed to be chance occurrences. However, lesions were seen only among the animals which died from the drug. In no instance did an animal survive with diabetes. Because of their early death, blood sugars were not obtained on the animals with lesions. Of those without lesions (series B) the blood glucose values in the rabbits are not regarded as significant. Four

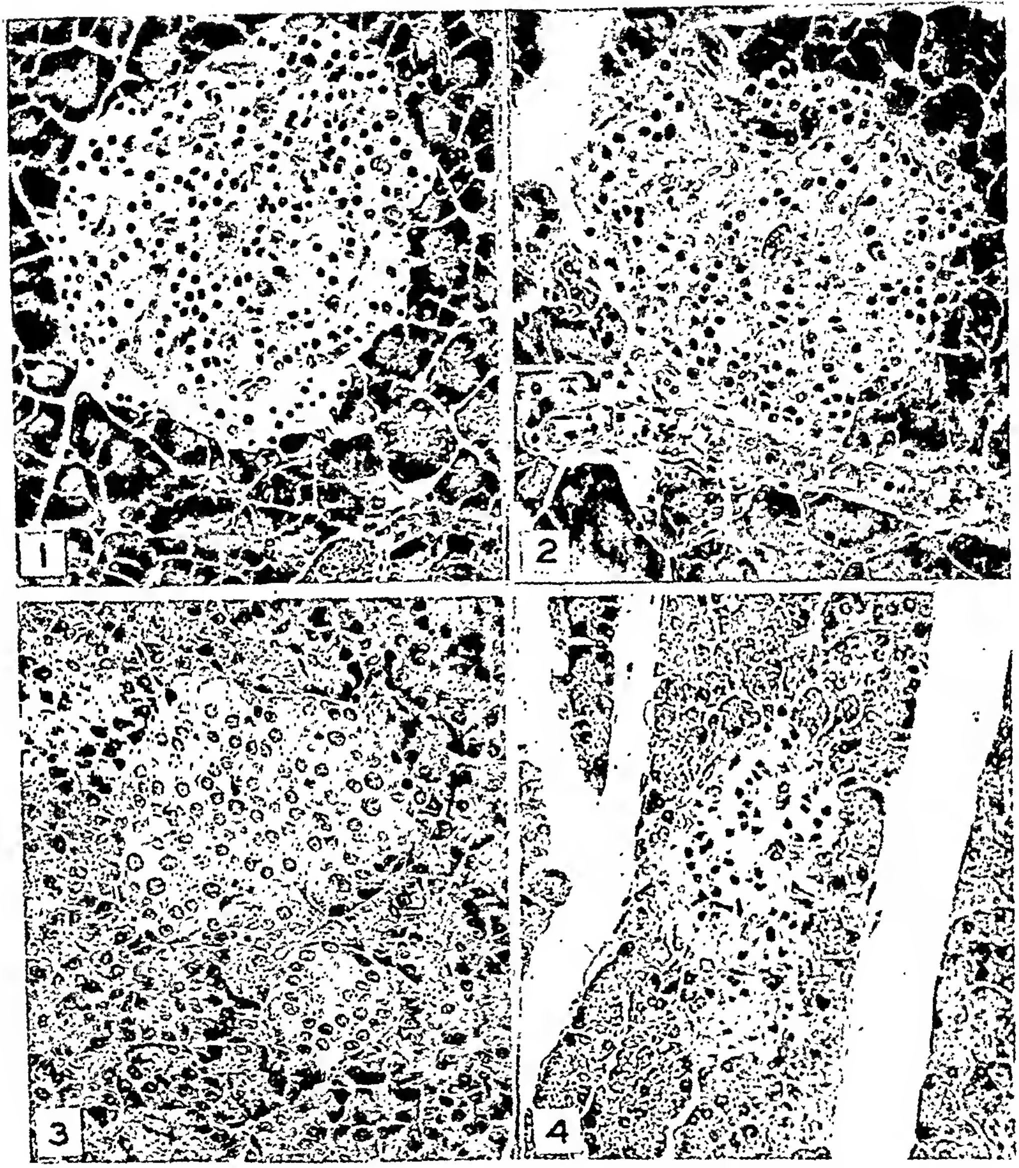

FIG. 1.
Island of Langerhans of Rat 3. Early necrosis is indicated by the pyknotic nuclei and loss of granularity of cytoplasm.

FIG. 2.
Island of Langerhans of Rat 17 showing early necrosis.

FIG. 3.
Normal island (rabbit).

FIG. 4.
Necrosis of island 11 hours after alloxan (rabbit).

of the rats without lesions had very high blood sugars indicative of diabetes but these animals were moribund at the time the blood sugars were determined. In all of 5 rabbits and 7 rats in which the kidney was examined microscopically, there was tubular necrosis of varying severity.

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	5	20–40	s.c.	12	102-130	15
III	6	20	i.v.	5	105-128	4–5
	4	20	s.c.	8	108-125	14
IV	6	10–20	i.v.	6	107-124	4–6
	6	20	s.c.	14	106-151	11–15
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* i.v. = intravenous; s.c. = subcutaneously.

TABLE II.
Effect on Pancreatice Islets of Compound VII: N[2(p-Diethylaminostyryl)-6-methyl quinoline] ethochloride.

Animal and No.	No. in group	Doses × days, mg/kg	Time of autopsy, days	Necrosis of islets	Blood sugar at autopsy
		A. Lesions observed.			
Rabbit 70		15 × 1 i.v.*	<1	+++	
" 76		30 × 1 i.v.	<1	+	
Rat 1		150 × 1 s.c.	1	+	
" 3		60 × 1 s.c.	1	+	
" 5		10 × 1 s.c.	1	+	
" 6		10 × 1 s.c.	1	+	
" 10		5 × 1 s.c.	1	+	
" 17		2 × 2 s.c.	1	+	
		B. No lesions present.			
Rabbits	8	10-30 × 1 i.v.	<1 to 5	0	121-178 (5 rabbits)
Rats	12	10 × 1 to 1 × 6 s.c.	1 to 6	0	101-702 (7 rats)

* i.v. = intravenously; s.c. = subcutaneously.

effect on the liver or other organs studied. The lungs, thyroid, thymus, adrenals, spleen were all grossly normal. Microscopic examination showed that the pancreas and islands of Langerhans were entirely normal. The liver, kidney and other organs showed no definite abnormality.

Of 28 animals tested with compound VII, 8 showed evidence of early necrosis of the islands of Langerhans. Table II summarizes these experiments, and Figures 1-4 illustrate the lesions in 2 of these animals. A normal island and the early necrosis from alloxan are presented for comparison. Early necrosis is shown by the shrunken, pyknotic nuclei and by the loss of normal granulation of the cytoplasm. There was no consistent relation of these lesions to dosage; they seemed to be chance occurrences. However, lesions were seen only among the animals which died from the drug. In no instance did an animal survive with diabetes. Because of their early death, blood sugars were not obtained on the animals with lesions. Of those without lesions (series B) the blood glucose values in the rabbits are not regarded as significant. Four extraction from horse or beef heart tissue, but the possibility of antigenicity has not been noted in any of the papers on cytochrome C seen to date, and only one brochure with a commercially available preparation suggests such a possibility.

Experimental. Three preparations of cytochrome C of different manufacturers were obtained; two were of equine origin, and one was of mixed bovine and equine origin. (For convenience, these preparations hereafter will be referred to respectively as CC-I, CC-II, and CC-III). All sensitizing doses were intraperitoneal. All shocking doses were via the dorsal penile vein.

Fourteen male guinea pigs were given 3 successive sensitizing injections of CC-I intraperitoneally at 2-3 day intervals. Group I, (9 animals), received 1 mg per dose, and Group II, (5 animals), received 5 mg per dose. In Group I, 3 weeks after the last sensitizing injection, 6 were challenged by the injection of CC-I via the dorsal penile vein. There was one fatal anaphylactic shock, 4 reactions with recovery, and one negative result. One week later the 3 of this group not previously tested were similarly challenged, and all died of anaphylactic shock. In Group II, all 3 animals challenged at 3 weeks died of anaphylactic shock, as did also the remaining 2 challenged at 4 weeks.

In a second experimental series, giving only 2 sensitizing injections of 2 mg per dose, 6 animals were sensitized to CC-I and 6 received similar doses of CC-II. Nineteen days later each group was divided so that 3 received the original sensitizing material, and the other 3 received the alternate product. None of the animals sensitized to CC-I showed any reactions, either to CC-I or CC-II. On the other hand, all animals initially sensitized to CC-II exhibited some degree of anaphylaxis when challenged with either CC-I or CC-II, and one death occurred with each material.

A third series of 9 guinea pigs was sensitized with CC-III, in 2 successive doses of 2.5 mg each. One month later 4 were challenged with the same material, 3 mg intravenously. None showed any shock symptoms. The remaining animals were challenged 5½ weeks after the last sensitizing dose, and none of these showed any reactions. Those tested at one month were again challenged 10 days later, and again no reactions were observed.

That the reactions observed in the first two series are due to primary antigenicity and not to a possible horse serum contamination was shown in a control series of 7 guinea pigs sensitized to horse serum. One animal given 0.75 cc horse serum died of anaphylactic shock. The 6 others received CC-I in doses from 3 to 10 mg and none showed any symptoms of shock. Exhaustion of the limited supply of the same lots of cytochrome C made it impossible to pursue further reciprocal testing with horse serum.

Discussion. The above experiments show that the clinical application of cytochrome C may be attended by a hazard similar to that experienced with horse serum. Attention should be drawn to some of the variations noted in the above experiments. Anaphylactic symptoms were more frequent and severe when the sensitizing doses were multiple and large, and when the incubation period was prolonged. When the number or quantity of sensitizing doses was reduced, anaphylactic symptoms diminished or were absent, so that no antigenicity was demonstrated under the experimental conditions. However, we have shown[5] that weakly antigenic materials, given a greater number of times for sensitization, and allowed a longer incubation period, may be shown to possess antigenicity not demonstrable by more commonly employed methods.

The possibility of a serious or even fatal anaphylactic reaction in the potentially sensitive human patient must therefore be considered when cytochrome C is administered intravenously, particularly if more than once, and testing precautions should be taken.

Summary. Guinea pigs sensitized with cytochrome C of equine origin when challenged with the same material, were shown to exhibit anaphylactic reactions, ranging to fatal, in a relatively large percentage of animals. Cross sensitivity could not be demonstrated between cytochrome C and horse tration of compounds I to VI caused no pancreatic damage requires the following comment:

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[5] Roth, L. W., Richards, R. K., and Shepperd, I. M., *Fed. Proc.*, 1948, 7, 105.

(a) The blood sugars were frequently above the normal level and this has been attributed to the technique used. When normal rabbits were handled in this manner blood sugars have been of this magnitude. There is a clear distinction between these values and those of rabbits with alloxan diabetes in which the blood sugars are characteristically high *i.e.*, 300 to 500 mg per 100 cc. The consistently negative urines provided a mass of evidence, not included in the tables, that these rabbits were not diabetic as a result of the quinoline compounds.

(b) The conclusion that these compounds are not diabetogenic is based on results in a single species. Although the rabbit was used by Sheehan[1] in the study of styryl quinoline, and although the rabbit is the animal most susceptible to alloxan, this limitation must be mentioned.

(c) The tendency to compare other toxic chemicals with alloxan may lead to errors in spite of every effort that has been made to observe diabetogenic activity. Thus, alloxan in similar sublethal doses would have caused diabetes with certainty, within the time limits of these experiments. A more insidious toxin could theoretically escape detection.

As far as we know, the results obtained with compound VII provide the first confirmation of Sheehan's[1] report that styryl quinoline caused injury of the pancreatic islands. As we gave styryl quinoline to only 4 animals our negative results with this compound (VI) might be accidental. This series was small because of Bailey's personal communication that he had not produced lesions of the islands or diabetes with styryl quinoline. The presence of lesions in the larger series of animals given a derivative of styryl quinoline (VII) is in general accord with Sheehan's[1] observations. He described "from memory" the consistent occurrence of islet necrosis after lethal doses of styryl quinoline (VI). In our series lesions occurred in the islets only after lethal doses but their occurrence was quite inconstant. In view of the absence of detailed protocols in the original report[1] further discussion is omitted.

Summary. Six quinoline derivatives have been administered intravenously and subcutaneously to rabbits and rats. There was no functional or anatomical evidence of damage to the islands of Langerhans or to any other organ under the conditions employed.

A seventh compound, N- 2-(p-Diethylaminostyryl)-6-methyl quinoline-ethochloride caused lesions of the islands resembling the early necrosis produced by alloxan. The lesions occurred in 8 of 28 animals tested. Islet necrosis was always associated with lethal dosage but death from the drug was not always accompanied by necrosis. Tubular damage in the kidney was found in all cases examined. Survival with diabetes was not observed.

16843

Potential Antigenicity of Parenterally Administered Cytochrome C.

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The separation and purification of cytochrome C by Keilin *et al.*[1] opened the field for its use in animal and human experimentation, in which encouraging results have since been claimed.[2-4] Its production usually involves extraction from horse or beef heart tissue, but the possibility of antigenicity has not been noted in any of the papers on cytochrome C seen to date, and only one brochure with a commercially available preparation suggests such a possibility.

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[1] Keilin, D., Hartree, E. F., and Hartree, F. R. S., *Proc. Roy. Soc. Med.*, 1937, **122**, 299.

[2] Proger, S., *Bull. New England Med. Center*, 1945, **7**, 1.

[3] Proger, S., and Decaneas, D. J., *Bull. New England Med. Center*, 1945, **7**, 149.

[4] Frank, H. A., Seligman, A. M., and Fine, J., *J. Clin. Invest.*, 1945, **24**, 435.

Experimental. Three preparations of cytochrome C of different manufacturers were obtained; two were of equine origin, and one was of mixed bovine and equine origin. (For convenience, these preparations hereafter will be referred to respectively as CC-I, CC-II, and CC-III). All sensitizing doses were intraperitoneal. All shocking doses were via the dorsal penile vein.

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Discussion. The above experiments show that the clinical application of cytochrome C may be attended by a hazard similar to that experienced with horse serum. Attention should be drawn to some of the variations noted in the above experiments. Anaphylactic symptoms were more frequent and severe when the sensitizing doses were multiple and large, and when the incubation period was prolonged. When the number or quantity of sensitizing doses was reduced, anaphylactic symptoms diminished or were absent, so that no antigenicity was demonstrated under the experimental conditions. However, we have shown[5] that weakly antigenic materials, given a greater number of times for sensitization, and allowed a longer incubation period, may be shown to possess antigenicity not demonstrable by more commonly employed methods.

The possibility of a serious or even fatal anaphylactic reaction in the potentially sensitive human patient must therefore be considered when cytochrome C is administered intravenously, particularly if more than once, and testing precautions should be taken.

Summary. Guinea pigs sensitized with cytochrome C of equine origin when challenged with the same material, were shown to exhibit anaphylactic reactions, ranging to fatal, in a relatively large percentage of animals. Cross sensitivity could not be demonstrated between cytochrome C and horse serum, but remains a possibility. Cross sensitivity to cytochrome C of different manufacture was demonstrated. The possible hazards of its use clinically are emphasized.

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[5] Roth, L. W., Richards, R. K., and Shepperd, I. M., *Fed. Proc.*, 1948, 7, 103.

16844

Assay of Anti-Pernicious Anemia Factor with *Euglena*.

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It was noted that massive growth of the algal flagellate *Euglena gracilis* depended on unknown growth factors present in crude casein but absent in certain plant proteins such as edestin and concanavallin A.[1] This factor was removed from casein by repeated isoelectric precipitation. It was subsequently found that alcoholic extracts of crude casein[‡] were active, and refined liver extract was recently reported to possess considerable activity.[2] It was found in the present investigation that this growth factor requirement was satisfied by a combination of crystalline antipernicious anemia factor (APA)[3] plus thiamine. These findings became the basis of an assay method for APA.

Trial of APA was suggested by the good agreement between the animal protein factor (APF) activity as measured with chicks, and the growth-promoting effect for *Euglena* of injectable liver extracts and of microbial APF concentrates. The latter were found to produce an hematopoietic response in pernicious anemia.[4]

Experimental. The organism used was *Euglena gracilis* var. *bacillaris*. The culture vessels were exposed to "daylight" fluorescent lamps at 25° to 31°C. The optimal temperature was 28° to 31°C; inhibition effects began to appear at approximately 32°C. At first, assays were carried out in 25-ml Erlenmeyer flasks covered with glass caps, and containing 10 ml of medium. It was later found convenient in routine assays to employ 100 x 13 mm tubes containing 2 ml of medium, illuminated from below. The light was supplied by 4, 40-watt lamps mounted side by side, 30 cm from the cultures. Light intensity did not appear to be a limiting factor at the levels of growth reported here. The basal medium is shown in Table I. The thiamine requirement was approximately 0.5 mμg/ml for half

TABLE I.
Composition of Basal Medium.

	Per l final medium (pH 6.5)	
$NH_4H_2PO_4$	0.8	g
Potassium citrate, monohydrate	0.2	"
$MgSO_4 \cdot 7H_2O$	0.2	"
Sodium butyrate	2.0	"
Monosodium glutamate	1.0	"
$CaCl_2$	0.1	"
$FeSO_4 \cdot 7H_2O$	20	mg
$MnSO_4 \cdot H_2O$	6	"
$CoSO_4 \cdot 7H_2O$	5	"
$ZnCl_2$	0.8	"
$Na_2MoO_4 \cdot 2H_2O$	1.0	"
$CuSO_4 \cdot 5H_2O$	0.08	"
Thiamine chloride	0.1	"

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[*] Haskins Laboratories. Aided by a grant from Lederle Laboratories Division, American Cyanamid Co.

[†] Lederle Laboratories Division, American Cyanamid Co.

[1] Hutner, S. H., *Arch. Protistenk.*, 1936, **88**, 93.

[‡] Generously supplied by the Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Provasoli, L., Hutner, S. H., and Schatz, A., PROC. SOC. EXP. BIOL. AND MED., in press.

[3] Smith, E. L., *Nature*, 1948, **161**, 638. A sample was kindly furnished by the Glaxo Laboratories, Ltd.

[4] Stokstad, E. L. R., Page, A., Pierce, J., Franklin, A. L., Jukes, T. H., Heinle, R. W., Epstein, M., and Welch, A. D., *J. Lab. Clin. Med.*, 1948, **33**, 860.

TABLE II.
Growth of Euglena in the Basal Purified Culture Medium with Various Supplements.

Basal medium used	APA factor added per ml of medium (mμg)	Other additions per ml of medium	Incubation period (hr)	Growth (optical density)
A	None	None	115	.04
"	.0015	"	"	.18
"	.005	"	"	.42
"	.015	"	"	.92
"	.05	"	"	1.34
"	.15	"	"	1.42
"	.5	"	"	1.40
"	None	0.05 mμl liver extr.	"	.14
"	"	0.15 " " "	"	.42
"	"	1.0 " " "	"	1.08
"	"	3.0 " " "	"	1.20
"	"	10 " " "	"	1.40
B	None	None	102	.07
"	.15	"	"	.21
"	"	0.05 mμg thiamine HCl	"	.34
"	"	0.15 " " "	"	.52
"	"	0.5 " " "	"	.97
"	"	1.5 " " "	"	1.32
"	"	5 " " "	"	1.30
"	.5	Vitamin mixture* without thiamine	92	.20
"	1.0	.05 μg nicotinic acid	"	.36
"	"	0.5 μg pantothenic acid	"	.35
"	"	5 μg pyridoxine HCl	"	.28
"	"	0.05 μg biotin	"	.30
"	"	0.5 μg pteroylglutamic acid	"	.24
"	"	5 μg *p*-aminobenzoic acid	"	.32
"	None	None	120	.13
"	1.0	0.5 μg thiamine HCl	"	1.40
"	None	0.5 μg thiamine HCl plus 0.5 μg thymidine	"	.12
"	"	0.5 μg thiamine HCl plus 1.5 μg thymidine	"	.11
"	"	0.5 μg thiamine HCl plus 5 μg thymidine	"	.12

A—Basal medium as in Table I.
B—Basal medium as in Table I except that thiamine was omitted.
* Riboflavin, niacin, pantothenic acid, pyridoxine, 0.5 μg each, biotin 0.05 μg.

maximum growth and 2.0 mμg/ml for maximum growth. Growth was practically complete in tubes in 4 days when a heavy inoculum was used. The inoculum was prepared by growing the organisms in 50-ml flasks containing 10 ml of basal medium supplemented with sufficient refined liver extract to allow about two-thirds maximum growth; one drop of a dense vigorous culture was then added to each tube or flask. Stock nutrient solutions were preserved by adding a mixture of *o*-fluorotoluene, 1, 2-dichloroethane, and *n*-butyl chloride.[5]

Results and Discussion. The results of some typical experiments are shown in Table II. It was found that about 0.01 mμg of APA factor per ml was required by *Euglena* for "half-maximum growth". This level is only approximately one-tenth as great as that required by *Lactobacillus leichmannii* 313.[6] Thymidine§ was inactive when tested up to

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[5] Hutner, S. H., and Bjerknes, C. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 393.

[6] Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176**, 1465.

§ We are indebted to Dr. D. W. Woolley for a sample of thymidine from the Levene collection, to Dr. J. O. Lampen for a sample prepared by him by the method of Klein,[7] m.p. 184.5° to 185.5° (uncorrected), and to Dr. W. Shive for a third sample.

[7] Klein, W., *Z. physiol. Chem.*, 1948, **255**, 82.

10 μg/ml. The lack of response to thymidine, in contrast to the effectiveness of thymidine for lactobacilli[8-10] draws attention to the value of comparative studies of *Euglena* and lactobacilli in exploring the functions of APA. Biochemical generalizations regarding these functions in various species should probably not be made solely on the basis of the observations with lactobacilli.

Experiments now in progress indicate that this preliminary assay medium is capable of further improvements.

Summary. The algal flagellate *Euglena gracilis* var. *bacillaris* was shown to exhibit a quantitative growth response to crystalline antipernicious anemia factor, using a chemically defined medium. Thymidine was inactive.

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[8] Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

[9] Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

[10] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

16845

Varying Effect of Thyroxine on Oxygen Consumption of Different Tissues.

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Some tissues from hyperthyroid animals preserve their increased metabolism after isolation from the animal. Tachycardia and increased oxygen consumption of the heart persist for hours.[1,2] Similarly, striated muscle and kidney tissue show a greatly increased oxygen consumption.[3] This shows that the increased metabolism is due to biochemical alterations in the cell, and not to nervous influences. It was thought by us that thyroxine might affect all actively metabolizing tissues in the same way. The experiments here reported show that this does not hold for brain cortex slices, which preserve a normal oxygen uptake despite marked hyperthyroidism in the intact animal. Liver, on the other hand, shows a peculiarly variable response.

Adult male albino rats (Donaldson strain) were used in all experiments. Thyroxine was injected subcutaneously in doses from 2.5 to 10 mg per kg every other day. The animals were sacrificed at from 7 to 21 days. Q_{O_2} of slices of gray matter was determined by the Warburg method, in 100% O_2, using phosphate buffer and glucose as substrate. In 26 experiments the Q_{O_2} of brain was found to be between 9.0 and 13.0 in all but 4 instances. In these 4 instances there was moderate increase ranging from 14.9 to 18.7. The mean Q_{O_2} for hyperthyroid brain was 11.5, which is exactly the same as that found in 13 experiments with normal brain (Table I).

In 14 experiments the Q_{O_2} of kidney slices from the same animals was determined. It was found to be greatly increased, averaging 36.1, or almost double the normal value. In 6 experiments the Q_{O_2} of small sheets of diaphragm muscle averaged 8.1, an increase of about 70% over the normal. The high rate of respiration of muscle and kidney tissue confirmed observations made by one of us some years earlier.[3]

In 13 instances the B.M.R. of the animal was followed, up to the time of sacrifice. This was done by the method of Tainter and Rytand.[4] All the animals were markedly hyper-

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[1] Lewis, J. K., and McEachern, D., Proc. Soc. Exp. Biol. and Med., 1931, **28**, 504.

[2] McEachern, D., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 287.

[3] McEachern, D., *Bull. Johns Hopkins Hosp.*, 1935, **56**, 145.

[4] Tainter, M. L., and Rytand, D. A., Proc. Soc. Exp. Biol. and Med., 1934, **32**, 361.

TABLE I.
Oxygen Consumption of Isolated Tissues from Normal and Hyperthyroid Animals.

	Q_{O_2} tissue (cu mm O_2/mg dry wt/hr)			B.M.R. animal (cc O_2/g/hr)		
	Brain	Diaphragm	Kidney	Initial	Final	Increase
Hyperthyroid series						
Mean	11.5	8.1	36.1	1.69	3.65	+116%
No. of exper.	(26)	(6)	(14)	(13)	(13)	(13)
Normal series						
Mean	11.5	4.8	22.5			
No. of exper.	(13)	(23)	(10)			
T.*	0	8.1	4.5			

* T. is the ratio of the difference to the probable error of the difference. It is only considered to be significant when the ratio is 3:1 or greater.

thyroid, as evidenced by the fact that their basal metabolic rates showed increases of between 84 and 201%. The mean increase for the group was +116%.

It is possible that brain metabolism may be somewhat increased during the first few days of thyroxine administration, and that later an anti-hormone effect may come into play. Of the 4 instances in which we obtained increases of oxygen consumption, 3 occurred in animals given thyroxine for only 7 to 9 days, whereas the majority received it for about 15 days. MacLeod and Reiss[5] found that after treatment of hypophysectomized rats with thyrotrophic hormone there was a moderate increase of oxygen consumption of brain slices between 5 and 8 days after commencement of treatment. After 16 days treatment the effect on brain was no longer apparent. Jandorf and Williams[6] found a reversion to normal in the B.M.R. of rats and the oxygen consumption of their isolated tissues after about 3 weeks treatment with thyrotrophic hormone. Certainly with thyroxine the increased oxygen consumption of muscle and kidney tissue, as well as that of the intact animal, is maintained over the period. This is not so for brain, and it may be an interesting example of homeostasis for a very select organ.

It is not clear why brain tissue fails to show the increase of oxygen consumption so characteristic of other tissues. It fits, however, with the observation of Gross and Leblond[7] who found that both white and gray matter showed minimal take-up of thyroxine labelled with radioiodine.

TABLE II.
Effect of Succinate on Oxygen Consumption of Liver Slices.

Hyperthyroid Q_{O_2}	Mean Q_{O_2} of liver		% Increase
	Glucose	Succinate	
0– 5	3.6	26.1	+625
5–10	7.7	53.2	+590
10–20	13.2	60.4	+357
Normal	11.2	39.4	+251

A curious phenomenon was found with hyperthyroid liver. The Q_{O_2} of normal liver is about 10.0, and liver from some hyperthyroid animals showed increases up to twice normal. Liver from other animals, however, gave Q_{O_2}'s that were practically nil. As shown in Table II, the Q_{O_2} was below 10.0 in 14 instances, in one case being as low as 1.3. In 13 experiments the Q_{O_2} was above 10.0, in one case reaching 21.3. This marked variability was at first attributed to some technical error but it soon became clear that this was not so. The phenomenon of very low Q_{O_2} was only found in the hyperthyroid series, and not with the normal controls which were run at the same time and which always gave Q_{O_2}'s ranging from 9.0 to 12.0. Duplicate runs were made on hyperthyroid liver and they agreed satisfactorily. Furthermore, the very low rates of respiration were only found with hyperthyroid liver and not with other tissues. The phenomenon may be due to liver damage or to the exhaustion of some substrate or enzyme system within the cells.

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[5] MacLeod, L. D., and Reiss, M., *Biochem. J.*, 1940, **34**, 820.

[6] Jandorf, B. J., and Williams, R. H., *Am. J. Physiol.*, 1944, **141**, 91.

[7] Gross, J., and Leblond, C. P., *J. Biol. Chem.*, 1947, **171**, 309.

When M/48 succinate was added at the end of the first hour it was found that the low-respiring liver slices were stimulated to remarkable activity, as shown in Table II. Here the results on hyperthyroid liver are considered in 3 groups, depending on the original Q_{O_2} of the tissue. The mean per cent increase for the lowest respiring group was 625%, considerably greater than for those tissues having a higher initial respiration. Suitably thin slices of tissue were chosen to meet the high O_2 consumption.[8] Increases of respiration were not as great as those obtained by the above author with starved liver since we used M/48 succinate instead of the M/25 optimal concentration. The differential increase between normal and hyperthyroid liver slices is clear, however. Our results show that the low-respiring liver slices may have difficulty in using glucose as substrate but are capable of using succinate.

Numerous experiments were carried out to try to pin down the cause of the low respiration in liver of some hyperthyroid animals, but without success. Three different types of diets were tried, one heavily supplemented with vitamins and minerals. These were Fox Chow, the diet of Sherman and Sandels[9] and that of McEachern.[3] The method of killing the animal was explored, using ether, gas and drowning in different experiments. The effect of fasting or of feeding right up to the time of sacrifice was investigated. None of the above factors appeared to play a role.

Rosenthal[8] found that the intensity of spontaneous respiration and the oxidation of lactate and pyruvate by liver slices is decreased in starved animals, but that the intensity of succinate oxidation is not influenced by starvation. He suggested that a decrease in the concentration of co-enzymes in the cell may be responsible since the oxidations of lactate and pyruvate, in contrast to the oxidation of succinate, are known to require co-enzymes. Peters and Rossiter[10] showed that rapid depletion of co-carboxylase occurs in the livers and other tissues of hyperthyroid rats fed upon a basal diet and that this defect is somewhat repaired by administration of thiamin. It is possible that hyperthyroidism may deplete the liver in some such way even when the animals are offered an adequate diet.

Summary. Cerebral gray matter of hyperthyroid rats does not show the increase of respiration shown by the intact animal or by diaphragm muscle or kidney tissue. Liver tissue shows a highly variable respiration in hyperthyroidism, sometimes being abnormally low. These low-respiring tissues are, however, greatly stimulated by the addition of succinate. It would seem that in hyperthyroid liver there may be a shortage of co-enzyme for the glucose-pyruvate cycle. The cytochrome-oxidase system, as judged by succinate response, remains intact or is enhanced.

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[8] Rosenthal, O., *Biochem. J.*, 1937, **31**, 1710.

[9] Sherman, H. C., and Sandels, M. R., *J. Nutrition*, 1931, **3**, 395.

[10] Peters, R. A., and Rossiter, R. J., *Biochem. J.*, 1939, **33**, 1140.

16846

Effect of Vagus on the Monophasic Action Potential of Auricular Muscle.

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A turtle auricular strip is deeply immersed in a large volume of Ringer's solution. Arrangements are made for unipolar recording of the action potentials. Non-polarizable electrodes are used in conjunction with a Cambridge All-Electric Electrocardiograph. A drop of mecholyl (acetyl-beta-methylcholine chloride; $1:10^4$) is placed on the tissue near the tip of the recording electrode. A stimulus is now applied to one end of the strip and a triphasic curve is recorded which looks like the membrane current curve of nerve. If one performs two successive integrations on this curve, or uses the graphical method recently described (Churney, Ashman, and Byer[1]), the monophasic action potential curve may be obtained. This monophasic curve resembles that of nerve and differs from that of the uninhibited auricle in at least two respects: (1) the descending limb characterizing the repolarization process is convex towards the time axis rather than concave; and, (2) its duration is shorter. By means of the suction electrode[2] one can experimentally verify this theoretically derived monophasic curve.

Another method for studying these phenomena, and one which yields additional information, is as follows. A cotton wick electrode soaked in isosmotic potassium chloride solution is fixed to the apex of the left auricle. The other electrode is placed either on the surface of the left auricle, or in the body cavity. The left vagus is exposed in the neck and prepared for stimulation.

Recording with the KCl-treated electrode on the auricle in conjunction with a remote electrode lends itself, we believe, to a very simple interpretation. Almost surely we are recording the potential changes of a ring of injured cells whose inner surfaces are in electrical communication with the KCl-treated lead. We find a ready interpretation of our results by treating the situation as though it were a single spherical cell, one side of which has its charges partially or entirely wiped out. On, or close by, this latter surface is the recording electrode; the other being remote.

The result of vagal stimulation on the auricular monophasic curve is shown in Fig. 1. In addition to showing clearly the effects mentioned already, the "spike" voltage is seen to be greatly reduced. (The quantitative aspects of the fall and recovery in the magnitude of the spike are of no consequence for the moment). So, even though there is only a very slight increase in the threshold of excitation (Ashman and Garrey[3]), provided the inhibition is partial, the action currents originating in the sinus give rise to potentials in the auricle which are below normal. This record, as well as others obtained from linear strips treated with mecholyl, shows that the monophasic action potential during vagal stimulation is not associated with a change in the level of polarization of the resting membranes. We have never observed a Gaskell effect.

The question now confronting us is this. Is the decrease in spike voltage real, or only apparent? More specifically, is it associated with a partial depolarization of the individual auricular muscle fiber, or does it result from the irresponsiveness of various fibers? Early investigators (Gaskell,[4] Rossbach,[5] and oth-

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* Address: College of Medical Evangelists, Loma Linda, Calif.

[1] Churney, L., Ashman, R., and Byer, E., *Am. J. Physiol.*, 1948, **154**, 241.

[2] Wiggers, H. C., *Am. J. Physiol.*, 1937, **118**, 333.

[3] Ashman, R., and Garrey, W. E., *Am. J. Physiol.*, 1931, **98**, 109.

[4] Gaskell, W. H., *J. Physiol.*, 1880, **3**, 48.

[5] Rossbach, M. J., *Arch. f. ges. Physiol.*, 1882, **27**, 197.

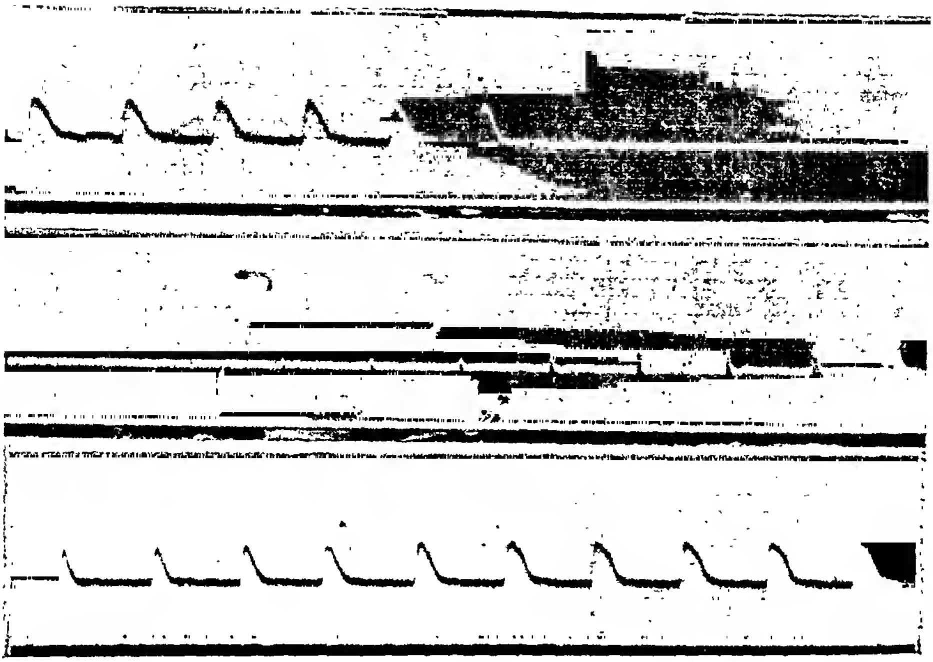

FIG. 1.
Effect of vagal stimulation on the monophasic action potential records of turtle auricular muscle. One electrode is on the KCl-treated apex of the left auricle, the other on the uninjured surface. The ventricle has been tied off to prevent its beating. Upper signal: left vagus stimulated faradically. Calibration: 3 millivolts.

ers) definitely assumed that the negative inotropic effect is the result of the diminished force of contraction of the entire syncytium, but without any real proof. See, too, the review of Adrian.[6] Concerning the reality of the modified spike potential itself there can be no doubt, since the theoretically predicted triphasic volume conductor curve is experimentally recorded.

Now there is no doubt that under some conditions mecholyl can cause blocking, and so the decrease in spike magnitude could be apparent only. However, at least four lines of evidence support the alternative viewpoint and are incompatible with the blocking concept. First, we recall the observation that there is a drastic change in the *form* of the monophasic action potential curve. Furthermore, there appears to be a strict association between the spike height and the shape of the recovery limb (Fig. 1). Not only is the convexity (towards the time axis) the more pronounced the less the apparent degree of depolarization, but the convexity appears *pari passu* with the decrease in spike magnitude following vagal stimulation, and does not become reversed during recovery until the spike is of full height, or very nearly so (Fig. 1). Secondly, we have confirmed the well known observation that vagal stimulation shortens the absolute refractory period of partially inhibited auricular muscle (Gilson[7]). There is some evidence that the less the spike height, or the shorter the duration of the electrical complex, the greater the percentage shortening of the absolute refractory period. With a decrease in spike amplitude to 17.9% of the original value, the "absolute refractory period" (as measured from the beginning of the spike to the signal of the applied stimulus) fell to at most 35.4% of its value for the unatropinized muscle. According to Asher and Scheinfinkel,[8] acetylcholine also shortens the absolute refractory period of nerve.

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[6] Adrian, E. D., *Ergeb. d. Physiol.*, 1933, **35**, 744.

[7] Gilson, A. S., Jr., *Am. J. Physiol.*, 1935, **112**, 610.

Thirdly, we cite the finding of Gilson[9] that there is a very great increase in accommodation of turtle auricular muscle following vagal stimulation. If one defines accommodation in the most general terms: namely, as an active resistance of the tissue to depolarization, then this phenomenon may be the excitation counterpart of the decrease in spike voltage and accelerated repolarization seen in the electrograms.

Finally, insofar as the duration of the monophasic action potential curve and the absolute refractory period are measures of the length of the depolarized zone,[10] we should expect this length to be considerably reduced by vagal stimulation. Some idea of the order of magnitude of this reduction may be calculated as follows. From diphasic records of linear strips in air the speed of conduction may be taken, roughly, as 0.5 m./sec., though this may be a slight underestimate. At room temperature (30°C) the duration of the monophasic curve of the uninhibited muscle is about 0.8 sec. The depolarized area, by far the greater part of which is in the state of repolarization, is therefore 40 cm long. The duration of the shortest monophasic curve following vagal stimulation is 0.04 sec. (Fig. 1), and, since there is no apparent change in conduction speed, its length is 2 cm.

Now, if the decrease in spike amplitude is real, the degree of depolarization of the inhibited muscle, as well as its length, must be very much less than that of normal, uninhibited muscle. If the extent of depolarization is not different, then, leading from the injured to the uninjured surface, a decrease in the diameter of the electrode on the uninjured surface should give a considerable increase in the percentage amplitude of the spike of the inhibited muscle. The fact that no such increase was found may be taken as further evidence that the decrease in spike height following vagal stimulation is associated with a partial depolarization of the individual heart muscle fiber.

Summary. The effects of vagal stimulation on the form of the monophasic action potential of auricular muscle are listed along with the technics for demonstrating them. Evidence is presented for the viewpoint that auricular muscle, following vagal stimulation, acts like a single cell capable of giving graded electrical, as well as mechanical, responses.

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[8] Asher, L., and Scheinfinkel, N., *Z. Biol.*, 1929, **88**, 540.

[9] Gilson, A. S., Jr., *Am. J. Physiol.*, 1939, **127**, 333.

[10] Lloyd, D. P. C., in Fulton's revision of Howell's Textbook of Physiology, Philadelphia, 1941.

16847

Failure of Rutin to Decrease the Mortality of Acute Ionizing Radiation Illness in Mice.*

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Rutin, a crystalline rhamnoglucoside of quercetin, has been reported to influence favorably the hemorrhagic syndrome of acute ionizing radiation illness in dogs.[1] It has been found to accelerate the healing of the skin lesions of rats induced by localized radiation injury.[2] It seemed desirable, therefore, to study the effect of rutin on the mortality of acute irradiation illness in mice.

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* Project NM 007 039, Report No. 16, Naval Medical Research Institute, Bethesda, Md., August 19, 1948.

† The opinions or assertions contained herein are the private ones of the authors and are not to be considered as official or reflecting the views of the Navy Department or the naval service at large.

[1] Rekers, P. E., and Field, J. B., *Science*, 1948, **107**, 16.

Materials and methods. White, Swiss, female mice, obtained from this Institute's colony 30 days after weaning, were fed ground Purina mouse pellets to which had been added 35 mg of ascorbic acid and 16 g of Brewer's yeast per 1000 g of food. They were approximately 7 to 8 weeks old when irradiated, having been isolated and observed for a period of at least 10 days before use in any experiment.

Rutin administration was commenced either 2 weeks prior to, or on the day of, irradiation. In one group receiving rutin *before irradiation*, the drug was given in the following doses: One hundred were given a saturated solution of rutin in water to drink (13 mg/100 cc). Ninety-nine were given a solution of 10 mg/100 cc in drinking water. One hundred received the saturated solution of rutin in the drinking water plus 20 mg of rutin per 100 g of ground food. In another group 99 animals received rutin beginning *on the day of exposure* (saturated solution in water plus 20 mg/100 g of food). All animals received the drug continuously for the 28 day post exposure observation period. The 196 control animals for the above groups received the same amount of radiation.

All irradiated control and treated mice of a given group were exposed simultaneously. The source of radiation was the angular beam of a 1000 KV, 3 ma, G.E. Industrial X-ray machine, the characteristics of which have been previously described.[3] The animals were exposed in specially designed cages constructed of 3/16" plywood. Each cage consisted of two tiers, each holding 25 mice, and conformed in shape to a segment of a circle with a one-meter radius. The tops and bottoms of the cages were made of window screening for ventilation. Details of the construction of the cages and of their placement are reported elsewhere.[4] Animals from each group were marked and uniformly distributed in the cages around the tube so that each segment of the circle contained a representative number from each group. By this method it was possible to irradiate 400 mice simultaneously.

The average output of the tube was 31.2 r/min. at the geometric center of the cages, which was 104.4 cm from the center of the target. The variation in irradiation output as measured at the center, front, back, top and bottom of each cage was not more than ±0.5 r/min. The exposure period was 22.5 minutes for all mice (a calculated dose of 702 r plus an estimated 3 r from the warm up of the tube, or a calculated total dose of 705 r ± 11 r.

All mice were weighed daily before and after the exposure to radiation. Cages were inspected in the morning and the evening for dead animals. A few gross pathologic examinations were performed. No microscopic examinations were made. In general the study consisted exclusively of survival and weight recordings.

Results. The administration of rutin alone to mice resulted in no evidence of toxicity. The usual clinical signs of radiation illness in both the control and rutin-treated *groups of irradiated* animals were identical. By the end of the 28-day observation period all surviving animals appeared well and were gaining weight.

In the first experiment there were 96 control mice and 99 experimental mice that received rutin for 2 weeks before irradiation. Mice began to die in the control group on the ninth day after exposure and in the rutin group on the eighth day. After the nineteenth day there were only scattered deaths. From this initial experiment it appeared that the mortality was increased from 40.6% in the control animals to 52.5% in the rutin-treated animals. The probability of these data being from the same distribution is between 0.10 and 0.20 as determined by the Chi

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[2] Griffith, J. Q., Jr., Anthony, E., Pendergrass, E. P., and Perryman, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 332.

[3] Tullis, J. L., Tessmer, C. F., Cronkite, E. P., and Chambers, F. W., Jr., Naval Medical Research Institute, Project NM 007-039, Report No. 3, Dec. 1947, in press, *Radiology*, 1949.

[4] Chapman, W. H., Sipe, C. R., Eltzholtz, D. C., Cronkite, E. P., Lawrence, G. H., and Chambers, F. W., Jr., Naval Medical Research Institute, Project NM 007-039, Report No. 14, July 1948.

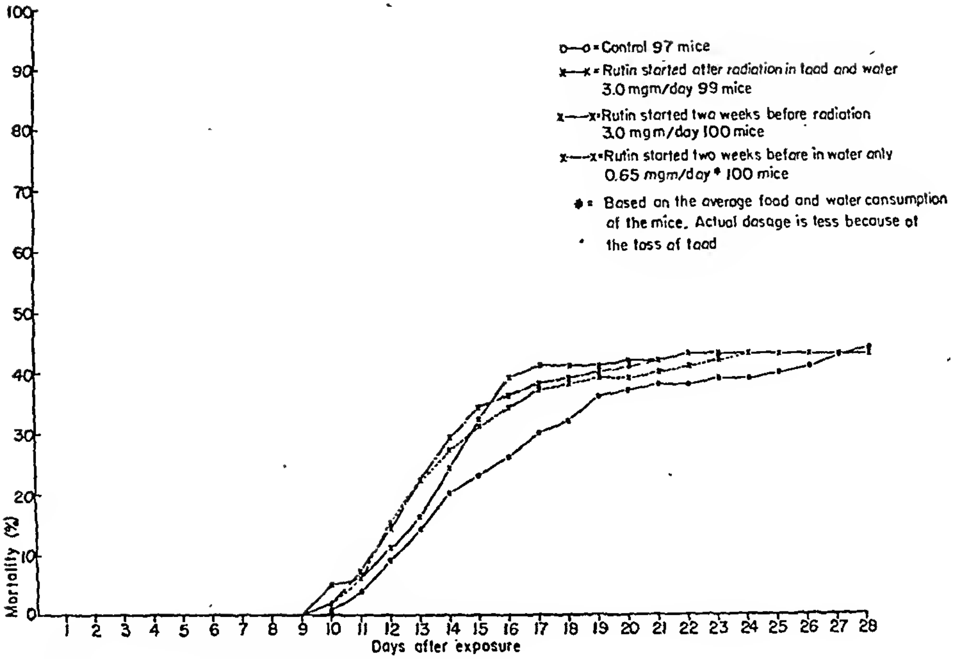

Figure 1 — Mortality curves of the control mice and the three rutin groups

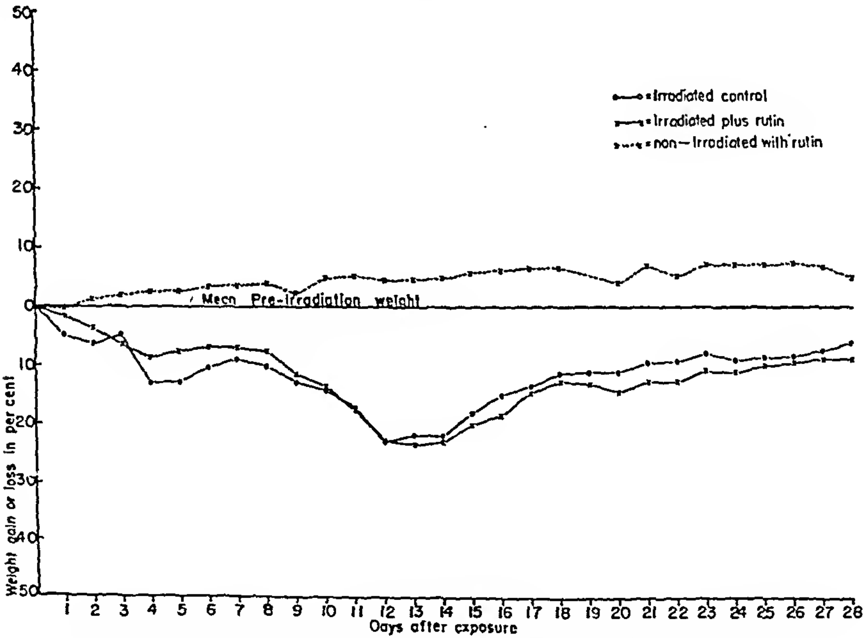

Figure 2.— Pooled average weight curves of mice in terms of percent loss or gain over the pre-experimental weight.

square method. Hence, the difference in results is not significant.

Fig. 1 shows that dosage and the time of administration did not significantly affect the results. The ultimate pooled mortality of the treated and non-treated mice on the 28th day was not significantly different ($0.70 > P_{diff} > 0.50$); however, on the 17th day the mortality of the rutin-treated groups was significantly greater than that of the non-treated groups ($0.05 > P_{diff} > 0.02$). This is further emphasized by the shorter average survival time of the combined rutin-treated animals that died (14.1 days) as compared to 15.4 days for the non-treated.

In Fig. 2 the pooled weights of all irradiated control mice, rutin-treated mice, and rutin-treated non-irradiated mice are plotted in terms of per cent gain or loss of original pre-irradiation weight. There was no significant difference between the irradiated control and irradiated rutin-treated mice. Maximum total weight loss was obtained by the 12th day after exposure when the mortality rate was at a maximum. The weight loss and mortality decreased simultaneously. However, scattered deaths did occur after the average weight began to increase. At the end of the observation period the average weight was still below that of the pre-irradiation period. The rutin, non-irradiated mice maintained a normal growth rate.

Autopsies of selected animals demonstrated the usual picture of irradiation illness in mice that has been adequately described in the past.[5]

Comment. The original attempt of Rekers and Field[1] to control the hemorrhagic syndrome of irradiation illness was based on the favorable clinical reports of rutin in the management of increased capillary fragility.[6,7] The results reported by Rekers and Field on the use of rutin in irradiated dogs were most encouraging. However, the *modus operandi* of rutin in controlling capillary fragility in general or in producing the favorable results of Rekers and Field in dogs is not known. It has been emphasized that rutin is less effective in the presence of an ascorbic acid deficiency.[7] Szent-Györgi and his co-workers[8] demonstrated that the crude glucosides of citrin could control increased capillary fragility of guinea pigs. It is probable, although not proved, that the active part of the citrin is rutin. The relationship of ascorbic acid and rutin in the maintenance of capillary fragility is not known. Apparently only man, monkey and guinea pig, in marked contrast to dogs and mice, are dependent upon diet for ascorbic acid. On this basis it is probably desirable to repeat this work on guinea pigs, an animal whose dietary requirements for maintenance of capillary integrity are more nearly like that of man. However, there are many factors which influence survival following exposure to radiation, and capillary integrity may not be the critical one. Many animals die with only minor evidence of hemorrhage.[9,10] The control of the coagulation defect with protamine and toluidine blue in dogs by Allen[11] prevented hemorrhage to a great extent but the animals *died nevertheless.* The extensive use of blood and penicillin by one of us did not significantly influence the survival of goats exposed to the atomic bomb.[9] The present work conclusively demonstrates that rutin in the doses used was of no value in improving the survival of mice. The data presented suggest that rutin may have been harmful inasmuch as all rutin-treated groups died at a significantly faster rate.

Summary and conclusions. 1. Rutin not only was of no value in improving the survival of mice simultaneously exposed to a dose of ionizing radiation in the lethal range, but significantly increased the rate at which the mice died.

2. It is considered desirable to repeat this type of investigation on animals with ascorbic acid requirements similar to man.

The authors wish to acknowledge their appreciation for the rutin kindly supplied by J. F. Couch, Eastern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture, Chestnut Hill Station, Philadelphia, Pa.

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[5] Ellinger, F., *Radiol.*, 1945, **44**, 125.

[6] Shanno, R. L., *Am. J. Med. Sci.*, 1946, **211**, 539.

[7] Couch, J. F., Krewson, C. F., Naghski, J., and Copley, M. J., U. S. Department of Agriculture, Bureau of Agricultural and Industrial Chemistry, April 1946, AIC-115.

[8] Armentano, L., Beutsath, A., Benes, T., Rusznyak, I., and Szent-Györgi, A., *Deutsche med. Wchnschr.*, 1936, **62**, 1325.

[9] Cronkite, E. P., Naval Medical Research Institute, Project NM 007 039, Report No. 10, July 1948.

[10] Prosser, C. L., *et al.*, *Radiology*, 1947, **49**, 299.

[11] Allen, J. G., personal communication.

16848 P

Increased Acetylcholine Sensitivity of Muscle by Acetone Magnification of the Rectus Test.

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It has been shown by Meng[1] that ketones and related substances sensitize the rectus muscle of the toad to acetylcholine (Ac). This sensitization is not correlated with inhibition of cholinesterase, and it is of the same degree whether the muscle is normal, eserinized or denervated. Hence the site of sensitization by these agents is said to be on the muscle. In connection with a study on the cholinesterase, we have found that acetone potentiates strikingly the action of Ac. The rectus muscle of the toad was recorded according to the standard technic.[2]

Fig. 1 compares the action of eserine and acetone on 2 recti of the same toad, showing the more striking potentiation by the latter. Note that neither eserine nor acetone alone produced any contraction, while addition of Ac which by itself elicited only a weak response, caused a very marked contraction. The action of acetone could be repeated in succession if it was given each time. The concentration of acetone used had no influence on the cholinesterase as was shown by the fact that addition of known amount of Ac to the acetone-treated and control muscle mince showed comparable degree of hydrolysis.

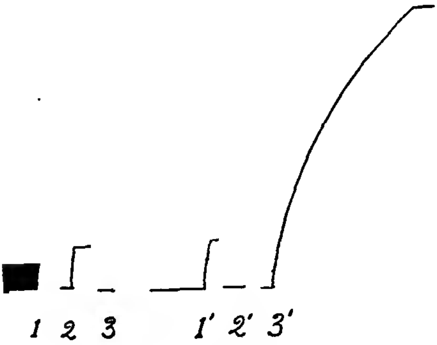

FIG. 1.
Comparing the sensitization of Ac by Es and acetone on the two recti of the same toad.

1. 1.38 γ Ac
2. 10 γ Es + 1.38 γ Ac } On right rectus
3. 10 γ Es

1' 1.38 γ Ac
2' 0.05 cc acetone } On left rectus
3' 1.38 γ Ac + 0.05 cc acetone

Muscle bath = 8 cc. Each contraction recorded for 3 min.

Feng and Lee[3] have demonstrated that acetone can reproduce practically all the diverse actions of eserine on the skeletal N-M system. In the present experiment, we have shown the synergistic action of acetone and Ac, or probably the action of the former on the surface membrane favoring more ready penetration of the latter to the contractile elements of the muscle. Incidentally, we have made practical use of this striking response of Ac plus acetone in two ways: First, as an additional collateral test for qualitative identification of Ac. It is comparable to the leech test.[4] Secondly, as a quantitative assay for Ac in the range of 0.05 to 0.5 γ. Usually 0.05 to 0.5 γ Ac given to a bath of 8 cc will not touch the rectus muscle of the toad. If it is given with acetone, a significant contraction will be produced. This magnifying action of acetone makes it very useful for quantitative assay of Ac in the weak concentration defined. It is necessary that same amount of acetone must be added to the standard and unknown.

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[1] Meng, C. W., *Chin. J. Physiol.*, 1941, **16**, 291.

[2] Chang, H. C., and Gaddum, J. H., *J. Physiol.*, 1933, **79**, 255.

[3] Feng, T. P., and Li, T. H., *Chin. J. Physiol.*, 1941, **16**, 317.

[4] Minz, B., *Arch. exp. Path. Pharmak.*, 1932, **168**, 292.

16849

Influence of Detergents on Egg-White Inhibition of Hemagglutination by Formolized Swine Influenza Virus.*

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During the past year investigations have been made of the capacity of hen's egg-white (EW) to inhibit hemagglutination by purified swine influenza virus and derivatives of this virus obtained by heating or treatment with formaldehyde.[1,2] Many of the studies involved the use of purified, formolized swine influenza virus[3] which had been prepared for vaccine and stored for approximately 4 years at 4°C. The use of this preparation as a reagent for titrating inhibitor was encouraged since vaccine hemagglutination was found highly susceptible to EW inhibition,[1,2] and since the preparation could be regarded to have become stabilized during the prolonged storage.

Recently, certain irregularities in the results of these inhibition titrations have made it necessary to search for the factors concerned. This study has revealed a strong influence of soaps and synthetic ionic detergents on the inhibition reaction.

Materials and methods. The formolized virus (vaccine) employed in the present work, as well as the method of preparation of egg-white and the methods for estimation of hemagglutinative or inhibiting capacities, have been described in a previous report.[2] The details of significant deviations from the routine procedures will be included in the description of the experiments given here.

Experimental. The irregularities stimulating the present work may be illustrated by the results of a set of duplicate anti-vaccine inhibition titrations carried out on three successive days by the methods previously described.[2] Table I shows a titration, I*a*, characterized by excellent duplication, *steep* endpoint gradient, and *high* conventional (++) inhibition titer. Titration I*b* is characterized by excellent duplication, *shallow* endpoint gradient, and *low* conventional titer; however, inhibition, as shown by the occurrence of agglutination numbers smaller than the maximal number (++++), is evident in high EW dilutions. In contrast with these, titration I*c* shows irregularities which preclude any useful statement of titer. The analytically smooth character of titrations I*a* and I*b* suggested that the irregularities evident in titration I*c* were to be attributed to a factor other than the technic of the titrations.

During the period in which the initial investigations[1,2] were carried out, the titration tubes were cleaned in a solution of "green" soap (Medicinal Soft Soap, U.S.P.) and a commercial synthetic detergent (Orvus, Procter and Gamble). Later, hot chromic acid ("cleaning" solution) was substituted for the detergent mixture. Since the onset of the analytical irregularities was approximately coincident with this change in cleaning method, it appeared possible that extraordinary disagreement of duplicates might have resulted from the inadvertent use of tubes cleaned in the two different ways for the two members of a duplicate.

The experiment recorded in Table II was performed as a test. Titration II*a* was carried out in tubes cleaned with hot chromic acid, washed thoroughly in tap water followed by distilled water, and dried in the oven. Titration II*b* was carried out in tubes cleaned first with acid and dried, as above, and then cleaned again in green soap-Orvus mixture,

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* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

[1] Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 312.

[2] Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 442.

[3] McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1945, **51**, 65.

TABLE I.
Three EW Anti-Vaccine Inhibition Titrations,* in Duplicate, Illustrating the Analytical Irregularities Which Stimulated the Present Investigation.

	Reciprocal of final EW dilution†						
Titration	1,600	3,200	6,400	12,800	25,600	51,200	102,400
I*a* (9/15/48)	0 0	0 0	0 0	+ +	++ ++	+++ +++	++++ ++++
I*b* (9/16/48	++± ++±	++± ++±	+++ +++	+++ +++	+++ +++	+++± +++±	+++± ++++
I*c* (9/17/48)	+++ ++	+++ +++±	+++± +	+± +++	+++± +++±	+++ +++±	+++± +++±

* Carried out at room temperature (28°C) with 4 HD (hemagglutinating doses) of vaccine. The EW dilutions and vaccine were incubated together for 40 minutes before RBC were added.

† Final EW dilution refers to the dilution of EW in the final reaction mixture including RBC.

TABLE II.
Influence of the Method of Cleaning Titration Tubes and of "Green" Soap on the EW Anti-Vaccine Inhibition Titration.

Titration		Reciprocal of final EW dilution									
	Cleaning method	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	†
II*a*	Acid	0 0	0 ±	++ ++	++± ++±	++± ++±	+++ +++	+++ +++	+++± +++±	+++± +++±	++++ ++++
II*b*	Detergent	0 0	± ±	+± ++	++ ±	++± ++	++± ++±	+++ +±	++ +++±	+++± +++±	++++ ++++
II*c**	Acid	0 0	0 0	0 0	0 0	0 0	0 0	0 0	+± +±	+++ +++	++++ ++++

* Green soap was present in 1:80,000 (V/V) final dilution in all tubes.

† Indicates mixtures devoid of EW.

with thorough rinsing in tap and distilled water. Titration II*c* was carried out in acid-cleaned tubes in the presence of a constant, known amount of green soap; the soap was used in a final dilution, 1:80,000 (V/V), well below the hemolytic limit (see below), which was 1:8,000. Other experimental factors were held constant. A volume of 0.25 ml of buffered saline (II*a* and II*b*) or 0.25 ml of a 1:10,000 dilution of green soap in buffered saline (II*c*) was added first to the tubes, followed by 0.25 ml of the EW dilutions, which had been prepared in bulk in acid-cleaned tubes, and then by 0.5 ml vaccine dilution containing the customary 8 HD (hemagglutinating doses) per ml. These mixtures were incubated for 35 minutes at room temperature (25°C), and 1.0 ml of 2% chicken RBC was then added. After an additional hour at room temperature, the readings were made in the usual manner.[4]

Result II*a* (Table II) shows excellent duplication, shallow gradient, and low conventional titer. II*b*, while similar on the whole to II*a*, shows several poor duplications. II*c* shows excellent duplication, steep end-point gradient, and high conventional titer. From these results it is evident that the irregularities present in II*b* may be explained as deviations from the pattern of II*a* in the direction of II*c*, and, accordingly, it is reasonable to attribute the irregularity of II*b* to an irregular residuum of soap in the tubes used in this titration.

An explanation may now be given for results of the sort shown in Table I. It may be supposed that titration I*a* was carried out in tubes which had been cleaned with detergent and in which the residuum of detergent was

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[4] Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

TABLE III.
Influence of "Green" Soap on the EW Anti-Vaccine Inhibition Titration; Dependence on Detergent Concentration.*

Reciprocal of final green soap dilution $\times 10^{-3}$	Reciprocal of final EW dilution									
	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	204,800	†
20	0	0	0	0	0	+	++±	+++±	++++	++++
40	0	0	0	0	0	+	+++	+++±	++++	++++
80	0	0	0	0	0	++	+++	+++±	++++	++++
160	0	0	0	0	0	++	+++	+++±	++++	++++
320	0	0	0	0	±	++	+++	+++±	++++	++++
640	±	±	+±	+±	++	++±	+++	+++±	++++	++++
1,280	+	+±	++	++	++±	++±	+++±	++++	++++	++++
†	+	+±	++	++±	++±	+++	+++±	+++±	++++	++++

* For this experiment, EW dilutions and soap dilutions were mixed first, then 4 HD vaccine was added to each tube; after 30 minutes at room temperature (27°C), RBC were added. Mixtures devoid of EW or soap are indicated (†), representing an infinite dilution of the respective reagents.

great enough and uniform enough to cause considerable and uniform depression of the agglutination; that titration *Ib* was carried out in tubes which had been cleaned with acid; and that titration *Ic* was carried out in a mixture of the two kinds of tubes.

It was important to investigate next the dependence of the inhibition on the quantity of detergent present. Anti-vaccine inhibition titrations were carried out in acid-cleaned tubes in the presence of graded amounts of green soap or Duponol PC (du Pont), a synthetic detergent consisting chiefly of sodium dodecyl sulfate, with about 8-10% sodium sulfate and sodium chloride, a small amount of sodium tetradecyl sulfate, and perhaps traces of other alcohol sulfates.[5] Since the two detergents gave essentially the same results, only those obtained with green soap are presented (Table III). Reference to Table III shows that a significant effect was detectable with green soap in a final dilution of 1:640,000; with Duponol PC, a significant effect was observed with the highest dilution tested, 1:1,920,000. Furthermore, as the amount of detergent was increased, the effect on the inhibition increased rapidly at first and then more slowly until a concentration was reached beyond which the change was very gradual; at the same time, the titration gradient progressed from a shallow gradient at low detergent concentration to a steep gradient at high detergent concentration. Approximately the same plateau inhibition titer was attained with the two detergents; expressed as the reciprocal of the final EW dilution, this titer was approximately 40,000.

A calculation shows that the amount of detergent needed for a great effect on the inhibition is of the order of the amount which might reasonably remain in detergent-cleaned tubes after rinsing. Duponol PC, in a concentration of approximately 1 γ per ml of final reaction mixture, corresponding to a final dilution of 1:1,000,000, exerts a great, almost maximal, effect. Since the reaction mixture has a volume of 2 ml, the total quantity of detergent needed for this effect is 2 γ. The internal surface area of a titration tube of the sort used in the present experiments is approximately 30 cm^2. On the assumption that the detergent molecules have an average molecular weight of 300 and that each molecule can occupy 20 sq. Å of glass surface when close-packed with other molecules, the quantity of detergent needed to form one complete unimolecular layer on the glass may be calculated as

$$\frac{(30 \times 10^{16})\ (300 \times 10^{6})}{20\ (6 \times 10^{23})} = 7.5\ \gamma.$$

The amount of detergent, 2 γ, needed for a great detergent effect is thus approximately ¼ of the amount which will cover the internal surface of a titration tube with a single, close-packed unimolecular layer. A similar result may be calculated for green soap if reasonable assumptions are made about the composition of this complex material.[6] Since it is known[7] that in concentrated solution detergents exist as aggregates (micelles) in equilibrium with unassociated ions, it is possible that aggregates, as well as free ions, are adsorbed to the glass during treatment with concentrated detergent solution and that portions of these aggregates survive the after-rinsing. In this way an amount of detergent greater than the amount needed to form a unimolecular layer could be made available. Furthermore, the recorded effective concentrations of detergent are probably in excess of the actual concentrations, since an undetermined amount may be supposed to have adhered to the walls of pipettes and vessels other than the titration tubes during the preparation and distribution of the detergent dilutions.

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[5] Personal communication from Dr. J. H. Shipp of the Fine Chemicals Division, E. I. du Pont de Nemours and Company, Inc., Wilmington, Del.

It was of interest next to determine whether the detergent effect on EW anti-vaccine titrations could be demonstrated with detergents of different kinds. For this experiment, 0.5 ml vaccine (4 HD) was first placed in each tube, and then 0.25 ml of a dilution of detergent well beyond the hemolytic limit (see below) was added, followed after 15 minutes by 0.25 ml of two-fold EW dilutions. After a further period of 20 minutes at room temperature, 1.0 ml 2% RBC was added, and the agglutination readings were made as usual after one hour. The *hemolytic limit* of each detergent was determined by mixing 1.0 ml 2% RBC with 1.0 ml of each of several two-fold dilutions of detergent; the endpoint was taken as the highest dilution of detergent at which hemolysis could be detected visually after one hour at room temperature. The detergents not previously tested included the anionic detergents sodium oleate (Fisher, U.S.P.), Orvus (Procter and Gamble), Aerosol OT (American Cyanamid), and Sodium "Lorol" Sulfate PT[†] (du Pont), a purified, essentially salt-free detergent, consisting chiefly of sodium dodecyl sulfate; the cationic detergent Zephiran chloride (Winthrop-Stearns); and the non-ionic detergent Tween 80[‡] (Atlas Powder). It was found that all of the ionic detergents exerted a great and strikingly similar effect on the EW anti-vaccine titrations; in other experiments, Zephiran chloride, which was noted to agglutinate RBC in high dilution of the detergent, gave irregular results which have not yet been further investigated. Tween 80, the only non-ionic detergent tested thus far, gave, in contrast with the other materials, a slight depression of inhibition, which has been confirmed but not further studied.

Discussion. A review of the data of Tables II and III shows that, in the range of concentrations in which they modify the EW inhibition of vaccine hemagglutination, the detergents, typified by "green" soap, do not inhibit hemagglutination by vaccine in the absence of EW; nor do they, in these concentrations, agglutinate RBC or cause hemolysis. On the other hand, EW, in the absence of detergent, gives evidence of *bona fide* inhibitor activity in high dilution, although the conventional (++) endpoint is reached in relatively low dilution. Indeed, examination of the results of many titrations indicates that, in general, EW and detergent together do not cause inhibition in EW dilutions appreciably beyond that dilution at which EW can be effective alone. Accordingly, we may provisionally interpret the detergent effect, which may be described as a sharpening of the inhibition endpoint, as an effect on the interaction of EW inhibitor and vaccine.

Summary. Investigation of the origin of irregular titration results has led to the observation that soaps and synthetic ionic detergents exert a great effect on the character of inhibition titrations involving purified, formolized swine influenza virus (vaccine) and the egg-white (EW) inhibitor of vaccine hemagglutination. The amount of detergent needed for such an effect is of the order of the amount which is sufficient to cover the internal glass surface of a titration tube with

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[6] Remington's Practice of Pharmacy, ninth ed., by Cook, E. F., and Martin, E. W., The Mack Publishing Company, Easton, Pa., 1948.

[7] Putnam, F. W., *Advances in Protein Chem.*, 1948, 4, 79.

[†] Obtained through the courtesy of Dr. J. H. Shipp of the E. I. du Pont de Nemours and Co., Wilmington, Del.

[‡] Kindly furnished by Dr. Hilda Pope of the Duke University School of Medicine.

TABLE III.
Influence of "Green" Soap on the EW Anti-Vaccine Inhibition Titration; Dependence on Detergent Concentration.*

Reciprocal of final green soap dilution × 10^{-3}	Reciprocal of final EW dilution									
	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	204,800	†
20	0	0	0	0	0	+	++±	+++±	++++	++++
40	0	0	0	0	0	+	+++	+++±	++++	++++
80	0	0	0	0	0	++	+++	+++±	++++	++++
160	0	0	0	0	0	++	+++	+++±	++++	++++
320	0	0	0	0	±	++	+++	+++±	++++	++++
640	±	±	+±	+±	++	++±	+++	+++±	++++	++++
1,280	+	+±	++	++	++±	++±	+++±	++++	++++	++++
†	+	+±	++	++±	++±	+++	+++±	+++±	++++	++++

* For this experiment, EW dilutions and soap dilutions were mixed first, then 4 HD vaccine was added to each tube; after 30 minutes at room temperature (27°C), RBC were added. Mixtures devoid of EW or soap are indicated (†), representing an infinite dilution of the respective reagents.

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[5] Personal communication from Dr. J. H. Shipp of the Fine Chemicals Division, E. I. du Pont de Nemours and Company, Inc., Wilmington, Del.

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Discussion. A review of the data of Tables II and III shows that, in the range of concentrations in which they modify the EW inhibition of vaccine hemagglutination, the detergents, typified by "green" soap, do not inhibit hemagglutination by vaccine in the absence of EW; nor do they, in these concentrations, agglutinate RBC or cause hemolysis. On the other hand, EW, in the absence of detergent, gives evidence of *bona fide* inhibitor activity in high dilution, although the conventional (++) endpoint is reached in relatively low dilution. Indeed, examination of the results of many titrations indicates that, in general, EW and detergent together do not cause inhibition in EW dilutions appreciably beyond that dilution at which EW can be effective alone. Accordingly, we may provisionally interpret the detergent effect, which may be described as a sharpening of the inhibition endpoint, as an effect on the interaction of EW inhibitor and vaccine.

Summary. Investigation of the origin of irregular titration results has led to the observation that soaps and synthetic ionic detergents exert a great effect on the character of inhibition titrations involving purified, formolized swine influenza virus (vaccine) and the egg-white (EW) inhibitor of vaccine hemagglutination. The amount of detergent needed for such an effect is of the order of the amount which is sufficient to cover the internal glass surface of a titration tube with a single, close-packed unimolecular layer. While the detergent effect shows a dependence on detergent concentration, there exists a broad range of detergent concentrations over which the effect on the inhibition is essentially constant. For reasons indicated, the effect of detergent has been provisionally interpreted as an effect on the interaction of EW inhibitor and vaccine.

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[6] Remington's Practice of Pharmacy, ninth ed., by Cook, E. F., and Martin, E. W., The Mack Publishing Company, Easton, Pa., 1948.

[7] Putnam, F. W., *Advances in Protein Chem.*, 1948, 4, 79.

[†] Obtained through the courtesy of Dr. J. H. Shipp of the E. I. du Pont de Nemours and Co., Wilmington, Del.

[‡] Kindly furnished by Dr. Hilda Pope of the Duke University School of Medicine.

The mechanism of the detergent effect is being investigated and will be the subject of a subsequent report.

16850 P

Assay for Antistiffness Activity with Guinea Pigs Depleted on Solid Rations.

WALDEMAR DASLER* AND CLIFFORD D. BAUER. (Introduced by Richard G. Roberts.)
From Nutrition Research Laboratories, Chicago.

Oleson *et al.*[1] have described a solid basal ration with which they depleted guinea pigs for the purpose of conducting assays for antistiffness activity. Their results, however, seem to indicate shortcomings in the assay method. Thus, 13% of their negative controls showed marked improvement and an additional 17% showed slight improvement in stiffness. Furthermore, their homologous series of ergostanyl esters showed wide, inexplicable differences in activity.

Petering *et al.*[2] have recently described an apparently satisfactory assay method in which they used a solid, semipurified diet and with which they claim to have confirmed the antistiffness activity of ergostanyl acetate.

We have confirmed the production of wrist stiffness in guinea pigs with solid rations. Both the Lederle basal ration described by Oleson *et al.*[1] and the Rockland stock diet for guinea pigs† were successfully used to produce wrist stiffness. On the basis of our results, however, it is questionable whether the condition produced in guinea pigs on these rations is identical to the Wulzen stiffness syndrome which develops in animals fed the skim milk diets.[3-5] In our hands, in fact, these commercial, pelleted rations proved unsatisfactory for the assay of anti-stiffness activity.

That the condition which develops in guinea pigs on the Rockland and Lederle rations is not identical to that which has been produced by the Oregon group on skim milk diets is indicated by the fact that in our animals the easily hydrolyzable phosphorus of the liver did not decrease as drastically as in the experiments reported by van Wagtendonk[5] and van Wagtendonk and Wulzen.[6] In fact, we found no correlation between wrist stiffness and the easily hydrolyzable phosphorus of the liver in animals fed these rations.

We have nevertheless attempted to determine the antistiffness activity of a number of materials in assays involving several hundred animals fed these diets. Among the substances assayed were the previously reported active compounds listed in Table I.

Animals having a 3+ stiffness[1] were used for the therapeutic assays which, except for the diet, were conducted by the method of van Wagtendonk and Wulzen.[4,6] Supplements were dissolved in cottonseed oil and adminis-

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* Present address: Department of Biochemistry, Chicago Medical School, Chicago, Ill.

[1] Oleson, J. J., Van Donk, E. E., Bernstein, S., Dorfman, L., and SubbaRow, Y., *J. Biol. Chem.*, 1947, **171**, 1.

[2] Petering, H. G., Stubberfield, L., and Delor, R. A., *Arch. Biochem.*, 1948, **18**, 487.

† Arcady Farms Milling Company, Chicago, Ill.

[3] Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, 1941, **133**, P500.

[4] van Wagtendonk, W. J., and Wulzen, R., *Arch. Biochem.*, 1943, **1**, 373.

[5] van Wagtendonk, W. J., *J. Biol. Chem.*, 1944, **155**, 337.

[6] van Wagtendonk, W. J., and Wulzen, R., *J. Biol. Chem.*, 1946, **164**, 597.

TABLE I.
Therapeutic Tests—Lederle Basal Ration.

Supplement	Dose 5x/week µg	No. of animals	Stiffness after 7 weeks			
			Improved	Slight or questionable improvement	No change	Worse
Ergostanyl Acetate[*]	5	19	1	2	2	15
	50	8	3	2	1	2
Ergostanyl Acetate[†]	5	18	2	1	2	13
	50	5	0	2	1	2
Natural anti-stiffness Factor[‡]	50	9	4	1	1	3
None	—	9	1	1	2	5
”	—	8	3	2	1	2

[*] Prepared by Dr. O. N. Breivik, Standard Brands, Incorporated.
[†] Supplied by Lederle Laboratories.
[‡] Supplied by Dr. W. J. van Wagtendonk.

TABLE II.
Ergostanyl Acetate[*]—8-Week Prophylactic Test—Rockland Diet.

Dose 7x/week, µg	No. of animals	Increase in stiffness		
		Marked	Slight	None
5000	10	4	1	5
500	10	6	3	1
5	6	3	2	1
0	15	6	2	7
0	15	10	3	2

[*] Supplied by Standard Brands, Incorporated.

tered orally. Because the results after 5 days were inconclusive, supplementation was continued. The assays were finally terminated after 7 weeks. The results are given in Table I. In this table weekly fluctuations in wrist stiffness are disregarded; only the over-all changes in stiffness which are manifest at the end of the 7-week period are indicated.

Prophylactic tests also were carried out with ergostanyl acetate on guinea pigs showing no stiffness and weighing 250-300 g at the start of the experiment. Our supply of the natural antistiffness factor was insufficient to permit its inclusion in this series. The results are shown in Table II. Some animals at all levels of ergostanyl acetate administration progressed through the whole range of wrist stiffness and, at the close of the eight-week experiment, had perfectly rigid wrists under the conditions of the test (1+ stiffness).

Summary and conclusions. Wrist stiffness was produced in guinea pigs by feeding two different commercial, pelleted rations. Both in therapeutic and in prophylactic tests, however, the untreated controls fared as well as the animals which received reputedly active supplements in amounts which have previously been shown to be more than adequate to give positive responses.[1,6] We interpret these results to indicate that (1) the mere production of wrist stiffness in guinea pigs does not necessarily yield animals satisfactory for assay purposes, that (2) the commercial, pelleted diets used by us and by Oleson *et al.*[1] are unsuitable as basal rations for the routine assay of the Wulzen antistiffness factor, and that (3) the activities of the steroids tested by Oleson *et al.* should be redetermined with animals depleted on more satisfactory basal rations.

16851

Inhibitory Action of Extracts of Mammalian Skin on Pigment Formation.

PETER FLESCH.* (Introduced by S. Rothman.)

From the Departments of Pharmacology and Medicine, Section of Dermatology, University of Chicago, Chicago, Ill.

Formation of melanin *in vitro* through oxidation of tyrosine by tyrosinase or by autoxidation of l-dihydroxyphenylalanine (dopa) is inhibited by skin extracts of rabbits[1,2] and of guinea pigs.[3,4] Rothman, *et al.*[5] have observed the inhibitory effect with extracts of isolated human epidermis. These authors advanced the theory that since they were able to counteract the inhibition with iodoacetamide, the inhibitory effect was due to the presence of sulfhydryl compounds in the epidermis. They suggested that pigmentogenic stimuli, such as ultraviolet rays, X rays, heat radiation and inflammatory processes caused pigmentation by oxidizing or destroying these inhibitory sulfhydryl compounds, thus enabling the enzyme to act on the pigment precursor.

The present work was carried out to investigate the nature and possible role of this inhibitory factor. The inhibitory action of aqueous extracts of human epidermis and of skin homogenates from rabbits was studied *in vitro*. In another series, rabbits were subjected to pigmentogenic stimuli and observations were made of the changes *in vivo*.

Methods. Aqueous extracts of human epidermis were prepared according to Rothman *et al.*[5] For preparation of extracts from rabbit skin, the hair on the back of the animals was clipped with scissors and on the bare skin two adjacent areas of equal size were mapped out with ink. One of the areas was covered with cloth, while the other was irradiated with an ultraviolet General Electric, RS, 275 W lamp. The distance varied from 6 to 12 inches and the exposure time from 5 to 15 minutes. Immediately after the irradiation the animal was killed. Skin samples were taken from the irradiated and non-irradiated areas. The subcutaneous fat tissue was removed and skin samples weighing from 0.3 to 0.5 g were frozen in dry ice and thawed rapidly several times in order to break up the cells. The pieces were minced and homogenized by means of glass homogenizers[6] with 2 ml distilled water in an ice bath, diluted with distilled water to a final concentration of 1 ml water for each 50 mg of tissue, placed in the ice box for 24 hours and centrifuged several times in the cold.

The inhibitory power of aqueous extracts of human epidermis and of the supernatant fluid of rabbit skin homogenates was determined by adding these extracts to buffered dopa solutions and by measuring the inhibition of melanin formation colorimetrically, as described elsewhere.[7] Sulfhydryl determinations were made with the ferricyanide method of Anson[8] in ultracentrifuged extracts and supernatant fluids of homogenates.

Results. In agreement with Rothman *et al.*[5] it was found that aqueous extracts of human epidermis inhibited the oxidation of tyrosine by tyrosinase and the autoxidation of dopa *in vitro*. As an index of the potency of the extracts the degree of inhibition of the autoxidation of dopa by 0.1 ml extract was chosen arbitrarily and the inhibition was expressed in percentages according to the formula:

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* American Cancer Society Fellow, 1948-49.

[1] Pugh, E. M., *Bioch. J.*, 1933, **27**, 475.

[2] Danneel, R., and Schaumann, K., *Biol. Zbl.*, 1938, **58**, 242.

[3] Schaaf, F., *Arch. f. Dermatol. u. Syph.*, 1938, **176**, 646.

[4] Ginsburg, B., *Genetics*, 1944, **29**, 176.

[5] Rothman, S., Krysa, H. F., and Smiljanic, A. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 208.

[6] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[7] Flesch, P., PROC. SOC. EXP. BIOL. AND MED., in press.

[8] Anson, M. L., *J. Gen. Physiol.*, 1941, **24**, 399.

$$\% \text{ Inhibition} = 100 \times \frac{(1\text{-colorimeter reading of tube containing extract})}{\text{colorimeter reading of control tube}}$$

Epidermis samples retained their inhibitory power for a considerable period of time when kept in the ice box before extraction. When the aqueous extracts were stored in the ice box, there was only slight diminution in the inhibitory effect. A fresh extract which caused 71% inhibition, showed at the end of 10 days 70%, and after 3 weeks 65% inhibition. Heating for 10 minutes in boiling water bath reduced the inhibitory effect by 10 to 40%. The inhibitory substance was completely dialyzed against distilled water through collodion membranes.

A few experiments were carried out with light brown extracts of 3 samples of Negro epidermis. No difference could be found between the extracts of epidermis from colored and white persons.

The inhibitory action of the extracts on dopa oxidation could be counteracted with p-chloromercuribenzoic acid, a specific sulfhydryl inhibitor. Cupric ion, a catalyst of sulfhydryl oxidation[9] and of the autoxidation of dopa,[10] also interfered with the inhibitory action of epidermal extracts. (Fig. 1).

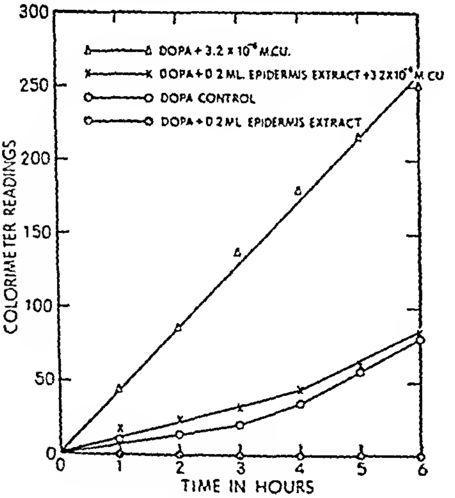

FIG. 1.
Inhibitory effect of human epidermal extract on autoxidation of dopa counteracted by cupric ions.

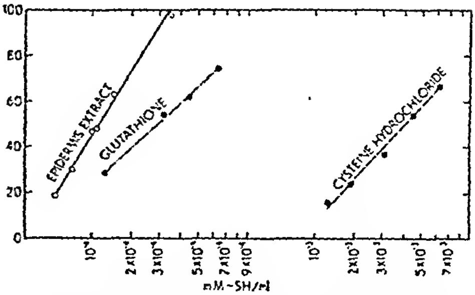

FIG. 2.
Relative inhibitory effect of cysteine, glutathione and extracts of human epidermis on autoxidation of dopa.

Estimation of the sulfhydryl content of the extracts gave values ranging from 4 × 10^{-5} mM/ml to 4 × 10^{-6} mM/ml. In most cases a correlation could be found between the sulfhydryl content of the extracts and their inhibitory power. When the -SH content was plotted on a logarithmic scale against the per cent inhibition, a straight line relationship was obtained. Such correlation was also found when glutathione (1.3 × 10^{-5} to 1.3 × 10^{-4} M final concentration) or cysteine hydrochloride (2.5 × 10^{-4} to 1.3 × 10^{-3} M final concentration) was added to dopa solutions. (Fig. 2). Calculated on the basis of the -SH content and in reference to 50% inhibition, the inhibitory effect of glutathione was about 13 times that of cysteine and the epidermis extracts were about 2.5 times more potent than glutathione.

When skin samples from human autopsy material were irradiated with ultraviolet light, no constant or reproducible differences could be observed between the -SH content of extracts of irradiated and non-irradiated epidermis. In order to eliminate the errors inherent in the preparation of human epidermis extracts (heating at 50°C for varying lengths of time, varying sizes of epidermal strips), living animals were irradiated and changes in the -SH content of the skin were studied in homogenates.

Colored rabbits are suitable for studies of pigmentation, because their hair is always

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[9] Bernheim, F., and Bernheim, M. L. C., *Cold Spring Harbor Symp. Quant. Biol.*, 1939, **7**, 174.

[10] Flesch, P., *J. Invest. Dermatol.*, 1948, **11**, 157.

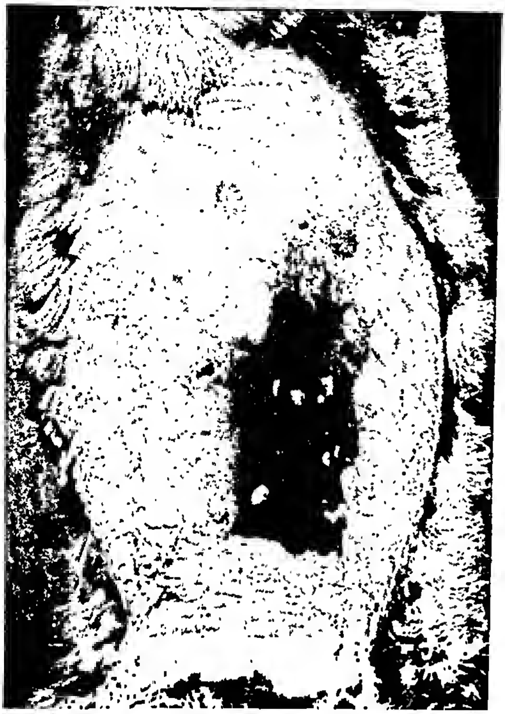

FIG. 3.
Pigmentation in grey rabbit after 12 days of rubbing daily for 1 minute with lanolin.

light colored near the skin. When the hair of colored rabbits is clipped and the bare skin subjected to irritation which leads to inflammation (heat, rubbing, ultraviolet light, etc.), the treated areas turn deeply pigmented after 1 to 2½ weeks and the regrowing hair becomes darker than it was before on the same area and than it is on the surrounding skin. The irritated areas are also characterized by exceedingly rapid regrowth of thick fur.[11-13] Clipping of the hair without irritating treatment leads primarily to an island-like regrowth of hair in pigmented areas[14] whereas hair growth in unpigmented spots is retarded.

In this laboratory one group of animals was rubbed daily for one minute with vaseline or lanolin over a clipped area on the back; the result in a grey rabbit after 12 days' rubbing is shown in Fig. 3. Another group of animals was irradiated daily for 10 minutes at a distance of 15 inches with the G.E. ultraviolet lamp. Pigment formation after ultraviolet irradiation was slightly more delayed than after rubbing. In experiments in which the -SH content of skin was determined, the rabbits were sacrificed immediately after a single irradiation was administered. In other animals one such treatment was found to cause pigmentation after 2 weeks latency period.

In 8 colored rabbits which were treated with ultraviolet light, the water extractable -SH compounds were markedly lowered in the aqueous extracts of homogenates obtained from irradiated skin samples as compared with similar extracts from non-irradiated skin. The average decrease was 53%. The results are summarized in Table I. Each result is the mean of three determinations, carried out in duplicate.

When the inhibitory power of these extracts was tested on the autoxidation of dopa, in 6 experiments the extracts obtained from irradiated skin showed 10 to 73% less inhibition than extracts from non-irradiated skin samples of the same animal. The average inhibition with extracts from irradiated skin was 29% and with extracts from non-irradiated skin 48%. (Fig. 4.)

Similar experiments were carried out on albino rabbits. In 6 experiments the amount of water-extractable -SH compounds did not show any significant change after irradiation. In 2 animals there was a decrease of 62 and 24% respectively.

On the basis of their -SH content, the extracts obtained from rabbit skin had about the same inhibitory power as aqueous extracts of human epidermis. No direct correlation could be found between the inhibitory power of the extracts and their -SH content.

Discussion. There are few data in the literature on the effect of ultraviolet light on sulfhydryl compounds of the skin. Keeser[15] found a marked reduction (50%) in the "reduced glutathione" content of rabbit skin after

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[11] Lutz, W., *Arch. f. Dermatol. u. Syph.*, 1917, **124**, 233.

[12] Linser, K., *Klin. Wchschr.*, 1926, **5**, 1490.

[13] Linser, K., and Kähler, H., *Klin. Wchschr.*, 1928, **7**, 116.

[14] Königstein, H., *Arch. f. Dermatol. u. Syph.*, 1923, **143**, 314.

[15] Keeser, E., *Arch. f. exp. Path. u. Pharm.*, 1932, **166**, 624.

TABLE I.
Effect of Ultraviolet Light on the -SH Content of Pigmented Rabbit Skin.

Rabbit No.	Hair Color	Exposure time and distance		-SH expressed in 10^{-5} mM in		% decrease
		min.	in.	Non-irradiated skin extracts	Irradiated skin extracts	
1	Grey	10	6	10 ± 0.3*	1.7 ± 0.3	83
2	,,	15	8	15 ± 0.4	7.9 ± 0.1	47
3	Red-brown	15	12	39 ± 2.4	30.0 ± 1.3	23
4	,,	7	12	19 ± 1.2	12.0 ± 0.7	37
5	,,	15	12	18 ± 0.8	8.4 ± 2.1	53
6	Black	15	12	24 ± 2.3	4.6 ± 1.1	81
7	,,	15	12	16 ± 1.4	4.8 ± 0.9	70
8	,,	15	12	24 ± 1.8	9.2 ± 2.3	62

* Standard deviation of the mean.

TABLE II.
Effect of Ultraviolet Light on the -SH Content of Albino Rabbit Skin.

Rabbit No.	Hair color	Exposure time		-SH expressed in 10^{-5} mM in	
		min.	in.	Non-irradiated skin extracts	Irradiated skin extracts
1	Albino	15	12	11 ± 0.9	12 ± 2.3
2	,,	,,	,,	18 ± 1.2	21 ± 3.2
3	,,	,,	,,	17 ± 0.8	18 ± 2.8
4	,,	,,	,,	28 ± 1.6	30 ± 4.0
5*	,,	,,	,,	41 ± 2.1	43 ± 2.6
6	,,	,,	,,	26 ± 1.8	24 ± 3.1
7	,,	,,	,,	22 ± 1.4	8.5 ± 1.3
8	,,	,,	,,	17 ± 0.6	13 ± 1.1

* Skin homogenate was extracted for 48 hours.

ultraviolet irradiation, while others[16] noted a slight decrease (3%) up to one hour after irradiation. The color of the rabbits used in all these experiments has not been mentioned.

The finding that ultraviolet light decreases the amount of water-extractable -SH compounds in pigmented skin, supports the assumption that pigmentation after irritative stimuli is at least partly due to a decrease in the -SH content of the melanoblast. The observation that a decrease in -SH content does not occur after ultraviolet irradiation of albino skin, can be explained by assuming that most of the radiation passes through the albino epidermis, as it is the case in the mouse,[17] while in the pigmented skin the melanin in the basal layer prevents the radiation from reaching the corium. Absorption of ultraviolet light is therefore much greater in pigmented than in non-pigmented epidermis which is the site of the -SH compounds and of the inhibitory action. [4,18-21]

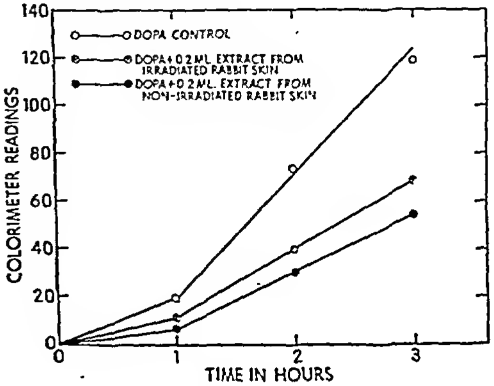

FIG. 4.

Inhibition of autoxidation of dopa with extracts of irradiated and non-irradiated rabbit skin.

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[16] Matusis, I. I., and Grechanovskii, V. P., *Bull. biol. med. exp. U.R.S.S.*, 1937, **3**, 489.

[17] Kirby-Smith, J. S., Blum, H. F., and Grady, H. G., *J. Nat. Cancer Inst.*, 1942, **2**, 403.

[18] Percival, G. H., and Stewart, C. P., *Brit. J. Dermatol. Syph.*, 1930, **42**, 215.

[19] Giroud, A., and Bulliard, H., *La kératinisation de l'épiderme et des phanères.* G. Doin, Paris, 1930.

[20] Kaye, M., *Bioch. J.*, 1924, **18**, 1289.

[21] Walker, E., *Bioch. J.*, 1925, **19**, 1085.

Summary. The inhibitory effect on melanin formation of aqueous extracts of isolated human epidermis and of homogenates of rabbit skin was found to be due to heat-stable, dialyzable, non-protein-like sulfhydryl compounds which were counteracted by cupric ions and p-chloromercuribenzoic acid. A direct relationship was found between the -SH concentration and the inhibitory power of extracts of human epidermis. Ultraviolet irradiation caused an immediate decrease in the amount of water-extractable -SH compounds of the skin of colored rabbits. No such decrease could be observed in albino animals. These findings support the previously advanced theory that pigment forming stimuli cause pigmentation by oxidizing or destroying the sulfhydryl compounds of the epidermis, whereupon the enzyme can freely act on the melanin precursor.

The author wishes to express his appreciation to Dr. Stephen Rothman for his valuable advice and interest in this work.

16852

Method for Determination of Sucrose and Sorbose in Blood and Urine.

CLARK D. WEST AND S. RAPOPORT.

From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine.

In this note is described a modification of the method of Hubbard and Loomis[1] for the determination of inulin which has proved satisfactory for the determination of sucrose and sorbose.

The method deviates from that described by these authors in the following ways.

The hydrochloric acid reagent is made by adding 5 volumes of concentrated HCl (sp. gr. 1.19) to one volume of distilled water. The resorcinol reagent is the same.

Plasma or serum filtrates are usually made in 1:20 dilution by precipitation with $Ba(OH)_2$ and $ZnSO_4$ according to the method of Somogyi.[2] Urine dilutions should be 1:100 or more. The one ml sample should contain between 30 and 160 μg of sucrose or between 20 and 150 μg of sorbose.

The procedure of Hubbard and Loomis is followed except for the following details. The test tubes are calibrated at 7.0 ml. After heating the mixture for 45 minutes in a water bath maintained at 70°C, 95% alcohol is added to the 7.0 ml mark, the solutions are mixed and are read in the Coleman spectrophotometer at a wave length of 530 mμ. In the case of sorbose, the density diminishes on standing; hence readings are made exactly 20 minutes after the end of heating.

Over the concentration ranges specified, the density was proportional to concentration for both sucrose and sorbose, sorbose being approximately 8% more chromogenic. Instead of running a blank we prefer to calculate a blank value by extrapolating the density of standards to zero content. In our experience the calculated blank density was usually less than 0.010.

Summary. The method of Hubbard and Loomis for the determination of inulin has been modified and adapted for the determination of sucrose and sorbose.

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[1] Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, 1942, **145**, 641.

[2] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

16853

Modification of Colorimetric Method for Determination of Mannitol and Sorbitol in Plasma and Urine.

CLARK D. WEST AND S. RAPOPORT.

From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine.

In this note is presented a modification of the method of Corcoran and Page[1] for the determination of mannitol which, in our hands, has proven simpler and more accurate than the original procedure. The method is applicable for the determination of sorbitol.

Reagents. 1. Periodic acid reagent: potassium periodate 0.0075 M in 0.25 M sulfuric acid.

2. Stannous chloride reagent: 0.035 M stannous chloride, reagent grade, in 0.33 M HCl. This reagent retains its strength for 4 days if kept in the refrigerator. After this time it results in blanks of high and variable density.

3. Chromotropic acid reagent. 0.15 g of chromotropic acid, purified by the method of Boyd and Logan[2] are dissolved in 20 cc of approximately 9 M sulfuric acid in a 200 cc volumetric flask. The contents are then made up to volume with concentrated (18 M) sulfuric acid. The first 20 to 30 cc of the concentrated acid should be added slowly, and with the flask in a cold water bath, to prevent heating the reagent. The density of the final reagent is approximately 0.010 at 570 mμ. It remains stable for many weeks if kept in the dark in a refrigerator.

4. Sulfuric acid, approximately 10.5 M.

Procedure. Mannitol-containing solutions should be preserved by the addition of a small amount of benzoic acid and kept at room temperature. Preservation in a deep freezing unit at —20°C may affect the determination by production of more color in the chromotropic reaction than corresponds to the liberation of 2 moles of formaldehyde per mole of mannitol.

Plasma filtrates are prepared as described by Somogyi.[3] Urines are diluted with water. The 2.0 cc sample should contain 7.0 to 40.0 μg of mannitol or sorbitol. The reagent blank consists of 2.0 cc of distilled water and is carried through the same procedure as the mannitol-containing solutions.

The procedure is carried out as follows: 2.0 cc samples of unknown, standards and water are pipetted into test tubes of 25 cc capacity. To each tube is added 0.5 cc of the periodic acid reagent and the contents are immediately and thoroughly mixed with a stirring rod. The tubes are then allowed to stand for 8 minutes at room temperature. The subsequent steps in the procedure should be carried out without interruption or delay.

After 8 minutes, 0.5 cc of stannous chloride reagent is added to each tube, the reaction mixture again well mixed, and the tubes are placed in a cold water bath. To each 5.0 cc of chromotropic acid reagent are added and the contents are mixed. It is advisable to add the chromotropic acid reagent to the blank before adding it to the mannitol-containing solutions. The tubes, with stirring rods in place, are immersed in a boiling water bath for 30 minutes. After removal and cooling, 10.0 cc of 10.5 M sulfuric acid are added to each tube, the contents are thoroughly mixed and the stirring rods removed.

The solutions are read in a Coleman spectrophotometer against distilled water at 570 mμ. The color is very stable, no change in density occurring if the tubes are permitted to stand for several hours before reading.

Calculations. Glucose, under the conditions described, is oxidized to formaldehyde to a slight and constant extent. By com-

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[1] Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1947, **170**, 165.

[2] Boyd, M. J., and Logan, M. A., *J. Biol. Chem.*, 1942, **140**, 279.

[3] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

Summary. The inhibitory effect on melanin formation of aqueous extracts of isolated human epidermis and of homogenates of rabbit skin was found to be due to heat-stable, dialyzable, non-protein-like sulfhydryl compounds which were counteracted by cupric ions and p-chloromercuribenzoic acid. A direct relationship was found between the -SH concentration and the inhibitory power of extracts of human epidermis. Ultraviolet irradiation caused an immediate decrease in the amount of water-extractable -SH compounds of the skin of colored rabbits. No such decrease could be observed in albino animals. These findings support the previously advanced theory that pigment forming stimuli cause pigmentation by oxidizing or destroying the sulfhydryl compounds of the epidermis, whereupon the enzyme can freely act on the melanin precursor.

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16852

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The method deviates from that described by these authors in the following ways.

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Summary. The method of Hubbard and Loomis for the determination of inulin has been modified and adapted for the determination of sucrose and sorbose.

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[1] Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, 1942, **145**, 641.

[2] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

TABLE I.
Distribution of Sheep Agglutinins.

	Strain							
	C57 Black		C3H		dba		Marsh-albino	
Titer	No.	%	No.	%	No.	%	No.	%
0	2	2	49	67.1	55	44.7	11	36.7
1	2	2	12	16.5	23	18.7	5	16.7
2	10	10	4	5.5	23	18.7	7	23.3
4	9	9	6	8.2	9	7.4	3	10
8	19	19	2	2.7	6	4.8	3	10
	Total	42		100		94.3		96.7
16	13	13	0		2	1.7	1	3.3
32	17	17	0		4	3.2	0	
64	15	15	0		1	0.8	0	
128	5	5	0		0		0	
256	4	4	0		0		0	
512	2	2	0		0		0	
1024	2	2	0		0		0	
	Total	58				5.7		3.3
Total No. of animals	100		73		123		30	

Sheep red blood cells, at least 24 hours old and not older than one week, were washed 3 times by centrifuging. The cells were not used unless the last supernatant was colorless. From the packed sheep cells a 2% suspension in physiologic solution of sodium chloride was prepared.

The test was set up as follows: Two drops of physiologic saline were added to each tube except the first. Two drops of serum were added to the first and second tube. After mixing, 2 drops of the second tube were transferred to the third tube, and so on. This gave serum dilutions of 1:2, 1:4, etc., which were continued according to the height of titer expected. One drop of the 2% sheep cell suspension was added to each tube and the rack was shaken. The tubes were incubated at room temperature for 2 to 3 hours. The reading of the agglutination was done grossly, and checked microscopically. Heavy "buttons" breaking up in large fragments were read as 3 plus; "buttons" breaking up in smaller grossly visible fragments, 2 plus; fine clumps visible under low-power magnification, 1 plus. The titer was given as the reciprocal of the highest dilution in which agglutination was found.

Results. Table I summarizes the titers obtained in the 4 inbred strains. Sheep agglutinin titers in C57 blacks reached by far higher values than those observed in any of the other strains. 58% of the C57 blacks presented titers of 16 or more, while among the C3H none of the titers exceeded 8; less than 6% of the dba animals and about 3% of the Marsh-albino exceeded this limit. 1024 was the highest titer observed in the C57 blacks; the highest titers in the C3H, dba and the Marsh-albino strains were 8, 64, and 16, respectively.

On the other hand, failure to agglutinate sheep erythrocytes in undiluted serum was found in only 2% of the C57 blacks, whereas 67.1% of C3H mice, 44.7% of dba animals, and 36.7% of Marsh-albinos showed titers of 0.

In order to study some of the properties of the antisheep agglutinins, pools of serum were prepared by sacrificing 9 to 11 animals of the C57 black strain. Preservation of the serum at 3-8°C for one month showed only insignificant changes in the agglutinin titer; after two months, usually a drop of the titer by 2 or more tubes was observed.

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[3] Davidsohn, I., *J.A.M.A.*, 1937, **108**, 289.

paring the densities of glucose-free mannitol standards with standards containing known amounts of glucose, the color production by glucose can be determined and correction for glucose content of the sample applied. In our experience the density per μg of glucose is 0.0024, as compared with 0.0142 per μg of mannitol. This value for glucose remains remarkably constant with varying proportions of glucose in the sample.

The density per μg of mannitol varies within ± 2% in the range of 10 to 30 μg of mannitol. For greater accuracy or for quantities beyond these limits, a calibration curve should be prepared.

Calculation of the mannitol content of the sample is as follows:

$$\frac{D_O - D_B - (K_G \times \mu g \text{ glucose in sample})}{K_m} = \mu g \text{ of mannitol in sample.}$$

Where D_O = observed density
D_B = density of blank
K_G = density per μg of glucose
K_m = " " " " mannitol.

Summary. A simple and accurate procedure for the determination of mannitol and sorbitol based on the method of Corcoran and Page has been described.

16854

Hemagglutinins in the Serum of Mice of Low and High Mammary Tumor Strains.

I. DAVIDSOHN AND K. STERN.

From the Mount Sinai Medical Research Foundation and The Chicago Medical School, Chicago, Ill.

The purpose of this communication is to report striking differences in the distribution of antisheep agglutinins in the serum of mice belonging to different inbred strains.

Preparatory to studying the response of inbred mice to antigenic stimulation, it became necessary to investigate natural hemagglutinins for sheep red cells, one of the antigens used. Gorer[1] reported the presence of isoagglutinins in mice, but no reference was found in the literature to natural heteroagglutinins in mice, although many investigations dealt with such natural antibodies in other species.[2]

Material. Healthy stock animals of 4 strains were used: 100 C57 black, 73 C3H, 123 dba, and 30 Marsh-albino animals.* The sex distribution was as follows: C57 black, 59 males and 41 females; C3H, 23 males and 50 females; dba, 32 males and 91 females; Marsh-albino, 9 males and 21 females. The age of the animals ranged from 10 weeks to 20 months, with the majority from 3 to 8 months. There was no apparent relation between the agglutinating ability of the mouse serum, and the sex or age of the animals, as represented in our material.

Technic. The animals were decapitated by a rapid stroke of sharp scissors. By holding the severed parts over a funnel, the blood was caught in small test tubes. The tubes were kept slanting until the blood clotted, left in the icebox for 18 to 24 hours, and then the serum was separated from the clots. The serum was inactivated in the water bath for 30 minutes at 56°C. The titration of the antisheep agglutinins followed the method used by the senior author in previous work.[3]

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[1] Gorer, P. A., *Cancer Research*, 1947, **7**, 634.

[2] (a) Kolle and Wassermann, *Handbuch der pathogenen Mikroorganismen*, 1929, **3**, 351, 784; (b) Shimidzu, T., *Tohoku J. Exper. Med.*, 1932, **18**, 526.

* The animals were either bred in this laboratory from parent stock received 3 years ago through the courtesy of Dr. A. Tannenbaum, Michael Reese Hospital, Chicago, or were received directly from Dr. Tannenbaum.

TABLE IV.
Antisheep Agglutinins and the Incidence of Spontaneous Mammary Cancer in Inbred Strains of Mice.*

Strain	Authors	Tumor incidence, %	Antisheep agglutinins Absent %	Antisheep agglutinins 16 and higher, %
C3H	Andervont ('41)	91.4	67.1	0
	Bittner ('43)	92.3		
dba	Korteweg ('36)	76.3	44.7	5.7
	Murray & Hoffman ('41)	64.5		
Marsh-albino	Murray & Hoffman ('41)	76.4	36.7	3.3
	Haagensen & Randall ('42)	76.4		
C57 Black	Little, Murray, & Cloudman ('39)	0.5	2.0	58.0
	Haagensen & Randall ('42) .	1.1		

* First 3 columns adapted from Walter E. Heston: Genetics of Mammary Tumors in Mice, in "A Symposium on Mammary Tumors in Mice," AAAS, 1945; p. 61, table I.

incidence of spontaneous mammary carcinoma, as recorded in the literature for the mouse strains furnishing the material of this study. It is apparent that the incidence of spontaneous mammary carcinoma in females in these strains is roughly inversely proportional to the presence and titers of the antisheep agglutinins. For instance, mice of the C57 black strain with extremely low mammary cancer incidence showed a very low percentage of serums lacking in sheep agglutinins and a high percentage of serums with titers of 16 and more. On the other hand, more than two-thirds of the serums of C3H mice, which possess the highest rate of tumor incidence, were completely lacking in agglutinins, and none of the animals so far examined showed a titer higher than 8. Animals of the dba strain, which develop mammary cancer quite frequently but less frequently than the C3H mice, lack sheep agglutinins in almost one-half of the cases, and less than 6% of the animals had titers of more than 8. Marsh-albinos, of which only a rather small number was tested, occupy a similar position in regard to tumor incidence; no agglutinins were found in more than one-third of the cases, and only 3.3% had titers higher than 8.

While no definite conclusions can be drawn from these findings at present, it may be hypothetically assumed that they are not coincidental but that the presence and the titer of the hemagglutinins are in some way connected with the genetic and other factors characterizing these mouse strains. In view of the fact that the incidence of spontaneous mammary carcinoma in the strains under discussion is determined by the milk factor and by hormonal influences in addition to genetic factors, those factors will have to be investigated.

The experiments on absorption of the agglutinins with guinea pig kidney suspensions showed that the agglutinins are not of the Forssman type; they also differed from the agglutinins found in serum of patients with infectious mononucleosis, since these latter are absorbed by beef red cells.[5] In this connection it may be pointed out that the position of the mouse in the Forssman system is not fully clarified. While older investigations placed this species into the "guinea pig group"[6] other workers presented contradictory evidence,[7] and more recent work showed differences between the true Forssman antigen and the antigens present in mouse tissues.[8] It is likely that especially the older investigations were carried out with heterozygous animals. For this reason studies on this

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[5] Davidsohn, I., *Am. J. Clin. Path.*, 1938, **8**, 56.

[6] Doerr and Pick, *Biochem. Z.*, 1913, **50**, 129 (quot. 2a).

[7] Davidsohn, I., *Arch. Path. and Lab. Med.*, 1927, **4**, 776.

[8] Brown, G. C., *J. Immunol.*, 1943, **46**, 325.

TABLE II.
Absorption Experiments.

	Titer		
		After absorption with	
	Unabsorbed serum	Guinea pig kidney	Beef cells
Pool I	32	20	20
,, II	64	40	40
,, III	64	40	40

Table II gives the results of absorption of the serum with suspensions of guinea-pig kidney and of beef erythrocytes. The preparation of these antigens followed the technic given by the senior author.[4] 0.1 cc of the serum was mixed with 0.5 cc of the antigen suspension, and the mixture was kept for 1 hour at room temperature, with repeated shaking of the mixture. Following centrifugation, the supernatant was set up simultaneously with the unabsorbed serum. For the calculation,. the dilution of the serum during the absorption was taken into consideration. Neither guinea-pig kidney nor beef red cells proved capable of removing the agglutinins from the serums to an appreciable degree.

Reading of the agglutination after increasing time intervals showed in some instances slightly higher titers after 2 hours of incubation as compared with the one hour titers; after 2 hours, the titers did not rise any further during observation for 24 hours. No significant change in titers was noted when the same serums were incubated at room temperature or 37°C. Occasionally, prolonged ice box incubation increased the titer by 1 or 2 tubes.

Instead of physiologic saline solution, albumin solution (20% bovine albumin) or serums of mouse, rat and rabbit were used as diluents for the serum to be tested and for the sheep cells. In the case of the animal serums, previous tests had shown the absence of antisheep agglutinins, or the serums were absorbed with sheep cells prior to use. In none of these experiments, did albumin or serum diluents raise the titer found with saline diluent; quite frequently, the titers in the albumin or serum dilutions were 2 to 3 tubes lower than the saline titers.

Table III lists agglutination tests which were set up with human O Rh-positive blood cells. The serum of 57 C57 blacks, of 61 C3H mice, and of 13 dba animals was tested. In marked contrast to the results obtained with sheep agglutinins, human red blood cells were clumped by less than 25 per cent of mouse serums and only in low titers. No appreciable difference was found between serums derived from animals of different strains. Nor was there any relationship between the simultaneously determined titer of sheep agglutinins, and the presence or absence of agglutinins for human erythrocytes.

In a few instances, also the agglutination of human erythrocytes of groups A and B (both Rh-positive) was tested with the serum of C57 black and of C3H mice. The number of experiments is too small to permit any conclusion. A cells showed a tendency to be clumped at a higher titer than O cells, but also here the titers were frequently far below those found in the same serum for sheep agglutinins.

Animals with spontaneous, transplanted and induced tumors were studied for the presence of hemagglutinins. The occurrence of antisheep agglutinins was not essentially different in tumor-bearing animals as compared with tumor-free animals of the same strains. A sufficiently large material to warrant definite conclusions is under investigation and will form the subject of a separate report.

Discussion. In Table IV the distribution of antisheep agglutinins is compared with the

TABLE III.
Agglutination of Human O Rh-positive Blood Cells By Mouse Serums.

	Strain		
Titer	C57 Blacks	C3H	dba
0	44	47	10
1	7	10	3
2	2	3	0
4	1	1	0
8	2	0	0
16	1	0	0
Total No. of Animals	57	61	13

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[4] Davidsohn, I., and Walker, P. H., *Am. J. Clin. Path.*, 1935, **5**, 455.

in blood.[3]

Separation of acetyl histamine from related substances. A mixture of 10 μg of histidine, 10 μg of histamine and 10 μg of acetyl histamine was applied to 2 paper strips (Strips 1 and 2, Fig. 1) and the individual substances to separate strips (Strips 3, 4 and 5, Fig. 1). The chromatograms were developed for 15 hours with butanol saturated with 10% ammonium hydroxide as the mobile phase and 10% ammonium hydroxide saturated with butanol as the phase at the bottom of the chamber. After brief drying, color was developed by drawing the strips through a solution prepared by mixing 10 ml of a solution of 0.125% p-bromoaniline in 0.1 N hydrochloric acid with 10 ml of 3.7% sodium nitrite solution and adding to this mixture 10 ml of a 20% sodium carbonate solution. Histidine, histamine and acetyl histamine appear as red bands which are well separated (Fig. 1). (A number of other histamine derivatives were chromatographed in a similar manner. All of those tested migrated much faster than acetyl histamine.)

$$\text{Approximate Rf values (Fig. 1)} \quad \frac{\text{Distance compound moves along paper}}{\text{Distance solvent moves}}$$

Histamine		Acetyl histamine	
Strip 1	.56	Strip 1	.71
" 2	.56	" 2	.71
" 5	.55	" 3	.71

The Rf values for histidine are difficult to estimate because of its slow migration rate with the solvent used.

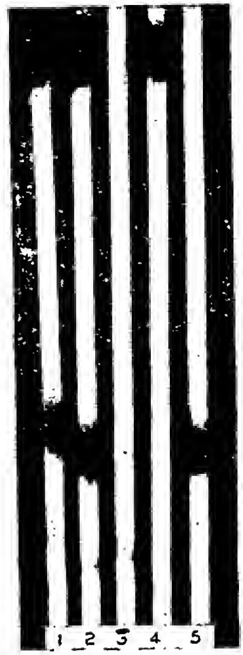

FIG. 1.

Separation of histidine, histamine, and acetyl histamine.

Strips 1 and 2: 10 μg histidine, histamine, and acetyl histamine applied as mixture.
Strip 3: 10 μg acetyl histamine.
Strip 4: 10 μg histidine.
Strip 5: 10 μg histamine.

Urinary excretion of the histamine derivative after feeding histamine to a dog. A normal urine sample was collected from a healthy male dog who was then given histamine diphosphate, equivalent to 200 mg of the free amine, dissolved in 250 ml of water by stomach tube. The dog was sedated with morphine and atropine prior to the administration of the histamine solution to avoid vomiting.[2] Freshly voided urine was collected thereafter at intervals up to 33 hours. From each sample of urine, 0.3 ml was diluted with water to 5 ml and 1.5 g of a salt mixture of 1 part $Na_3PO_4 \cdot H_2O$ and 6.25 parts anhydrous Na_2SO_4[4] was added. Two ml of butanol were added to the mixture and the tubes shaken for 20 minutes. After centrifugation, the butanol layer was applied to the paper strips by capillary pipettes.[5] Suitable controls were prepared by applying to strips extracts obtained from urine samples to which acetyl histamine and histamine had been added and by applying the pure substances to separate strips. The chromatograms were developed in the manner described in the foregoing experiment and uniformly showed bands in a position identical with that occupied by pure acetyl histamine on the control strips (Fig. 2). The chromatogram representing normal problem are being carried out in this laboratory using inbred strains.

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[3] Urbach, K. F., PROC. SOC. EXP. BIOL. AND MED., 1948, 68, 430.

[4] McIntire, F. C., Roth, L. W., and Shaw, J. L., *J. Biol. Chem.*, 1947, 170, 537.

[5] Urbach, K. F., in press.

Previous work on the carmine storage in mice of inbred strains[9] demonstrated an inferior reticulo-endothelial storing ability in animals of the C3H strain as compared with C57 blacks. Although the origin and the significance of natural antibodies, such as the antisheep agglutinins studied in the present report, is not yet understood, there is considerable evidence in support of the part played by the reticulo-endothelial system in their production. In addition to the previously reported depression of the storing capacity of reticulo-endothelial tissues, the present study suggests impairment of the reticulo-endothelial system in relation to the production of natural antibodies in some mouse strains with high spontaneous mammary cancer incidence as compared with the resistant C57 black strain.

Summary. Heteroagglutinins for sheep erythrocytes were studied in the serum of 4 inbred mouse strains. Antisheep agglutinins were present much more frequently and in significantly higher titers in C57 black than in C3H, dba, and Marsh-albino strains. Some properties of the sheep agglutinins were studied. No such differences were found in the distribution of heteroagglutinins for human red cells in the different strains. The possible significance of these findings was discussed.

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[9] Stern, K., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 315.

16855

Nature and Probable Origin of Conjugated Histamine Excreted After Ingestion of Histamine.

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Anrep and coworkers reported that a histamine derivative appeared in the urine of various animals and a human subject after oral administration of histamine. This histamine derivative was inactive when tested on the isolated guinea pig ileum, but after acid hydrolysis of the compound a typical histamine response could be elicited. Injection of histamine was not followed by increased excretion of the derivative. Liver or intestine were suggested as likely sites for the conjugation of the ingested histamine.[1] Rosenthal and Tabor recently made the important observation that the chemical behavior of the urinary histamine derivative resembled that of acetyl histamine [4(β-acetyl aminoethyl) imidazole].[2]

The purpose of the present investigation was to test whether or not the histamine derivative is actually identical with acetyl histamine and to discover the origin of the compound in the body. The experiments described below present evidence, obtained by paper chromatography, for the identity of the histamine derivative with acetyl histamine and show that this compound appears in the feces as well as in the urine after oral administration of histamine. It is further shown that histamine is rapidly acetylated when added to fresh but not when added to previously autoclaved feces. Lastly, some common fecal microorganisms are shown to produce traces of acetyl histamine from histamine. The evidence obtained suggests that ingested histamine is acetylated within the intestinal contents from which it is partially absorbed and excreted by the kidneys. The methods employed are modifications of those previously developed for the identification of histamine

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[1] Anrep, G. V., Ayadi, M. S., Barsoum, G. S., Smith, J. R., and Talaat, M. M., *J. Physiol.*, 1944, **103**, 155.

[2] Rosenthal, S. M., and Tabor, H., *J. Pharm. and Exp. Therap.*, 1948, **92**, 425.

Strip 1—Normal urine.
" 2—Urine 22 hr after histamine administration.
" 3—Urine 33 hr after histamine administration.
" 4—Urine 33 hr after histamine administration, 20 μg acetyl histamine added.
" 5—Normal urine, 10 μg histamine and 10 μg acetyl histamine added.
" 6—Histamine and acetyl histamine (control).

FIG. 3—Chromatograms of human urine after oral administration of histamine.
Strip 1—Normal urine.
" 2—Urine 1½ hr after histamine administration.
" 3—Histamine and acetyl histamine (control).
" 4—Urine 3½ hr after histamine administration.
" 5—Urine 6 hr after histamine administration.
" 6—Urine 8 hr after histamine administration.
" 7—Urine 20 hr after histamine administration.

FIG. 4—Chromatograms of hydrolysis of acetyl histamine.
Strip 1—Acetyl histamine before hydrolysis.
" 2—Acetyl histamine after hydrolysis.
" 3—Histamine (control).

FIG. 5—Chromatograms of human stool after oral histamine administration.
Strip 1—Normal stool.
" 2—Stool 6 hr after histamine administration.
" 3—Stool 20 hr after histamine administration.
" 4—Normal stool plus acetyl histamine and histamine (control).

FIG. 6—Chromatograms of effect of histamine addition to bowel loops and bowel contents of a dog.
Strip 1—Washed ileum loop immediately after histamine addition.
" 2—Washed ileum loop incubated 4½ hr after histamine addition.
" 3—Ileum contents immediately after histamine addition.
" 4—Ileum contents incubated 4½ hr after histamine addition.
" 5—Washed colon immediately after histamine addition.
" 6—Washed colon incubated 4½ hr after histamine addition.
" 7—Colon contents immediately after histamine addition.
" 8—Colon contents incubated 4½ hr after histamine addition.
" 9—Acetyl histamine (control).
" 10—Colon contents before histamine addition.

FIG. 7—Chromatograms of effect of histamine addition to human stool.
Strip 1—Normal stool.
" 2—Histamine and acetyl histamine (control).
" 3-9—Stool immediately, 1 hr, 4 hr, 8 hr, 24 hr, 30 hr, and 55 hr after histamine addition and incubation.
" 10—Normal stool 24 hr after incubation.

FIG. 8—Chromatograms of effect of autoclaving human stool on acetyl histamine formation.
Strip 1—Autoclaved stool.
" 2—Autoclaved stool immediately after histamine addition.
" 3—Autoclaved stool incubated 4½ hr after histamine addition.
" 4—Nonautoclaved stool.
" 5—Nonautoclaved stool immediately after histamine addition.
" 6—Nonautoclaved stool incubated 4½ hr after histamine addition.
" 7—Acetyl histamine (control).

FIG. 9—Chromatograms of effect of addition of histamine to pure cultures of *Aerobacter aerogenes* and *E. coli*.
Strip 1—Aerogenes culture immediately after histamine addition.
" 2—Aerogenes culture incubated 40 hr after histamine addition.
" 3—Histamine and acetyl histamine (control).
" 4—*E. coli* culture immediately after histamine addition.
" 5—*E. coli* culture incubated 4½ hr after histamine addition.
" 6—Histamine and acetyl histamine (control). (Traces of acetyl histamine at arrows. On Strip 5 at least 2 unidentified bands appear below the acetyl histamine).

urine shows the presence of a small amount of the histamine derivative (Strip 1, Fig. 2).

Approximate Rf values (Fig. 2)

Histamine		Urinary histamine derivative		Control acetyl histamine	
Strip 5	.55	Strip 1	.73	Strip 5	.74
" 6	.57	" 2	.73	" 6	.73
		" 3	.73		
		Urinary histamine derivative with acetyl histamine added			
		Strip 4	.73		

Urinary excretion of the histamine derivative after ingestion of histamine by a human subject. After collecting a sample of normal urine, a gelatin capsule containing 100 mg histamine diphosphate was given by mouth to a human subject (K.F.U.). Urine was collected at frequent intervals after the ingestion

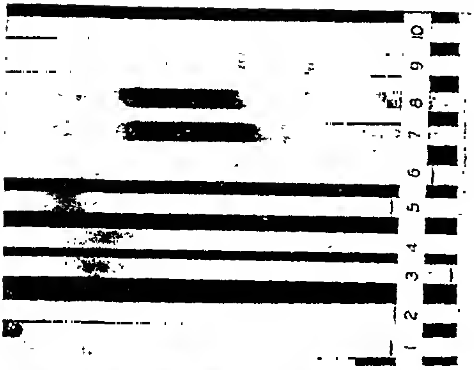

FIG. 6

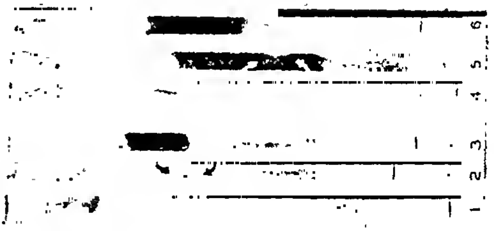

FIG. 9

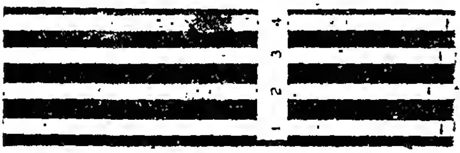

FIG. 5

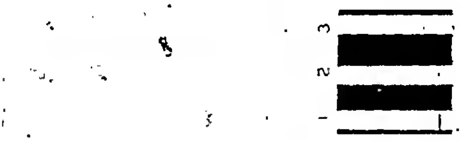

FIG. 4

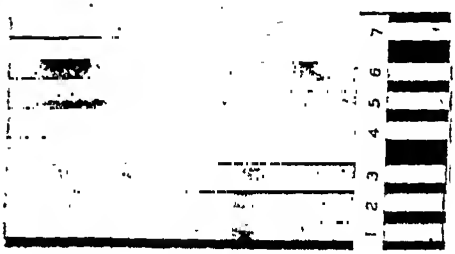

FIG. 8

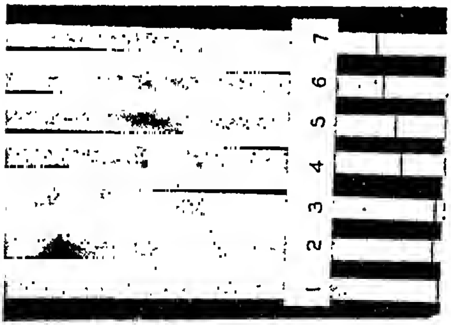

FIG. 3

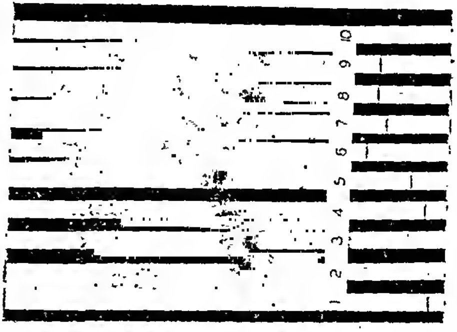

FIG. 7

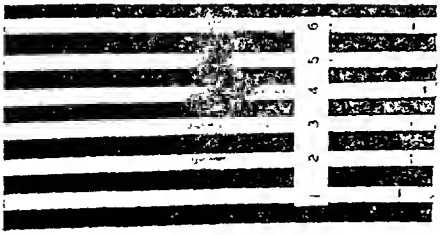

FIG. 2

FIG. 2–9.

(All upper ends of the strips have been cut. The pencil lines on the lower ends of some of the strips indicate how far the solvent has advanced. On all chromatograms the upper bands are due to histamine and the lower due to acetyl histamine (histamine derivative) unless otherwise indicated).

FIG. 2—Chromatograms of dog urine after oral administration of histamine.

faint trace of histamine. Strip 4 represents normal stool with histamine and acetyl histamine added.

Approximate Rf values (Fig. 5)

Histamine		Histamine derivative in stool		Control acetyl histamine	
Strip 2	.57	Strip 2	.71		
Strip 3	.57	” 3	.72		
Strip 4	.57			Strip 4	.70

Formation of the histamine derivative after addition of histamine to isolated intestinal loops and bowel contents of a dog. The appearance of the histamine derivative in feces, described in the previous experiment, could be due to two different mechanisms. Either the histamine derivative is formed inside the intestine or histamine is absorbed from the intestine, changed and re-excreted into the contents of the bowels. Since the histamine derivative had not been observed in the urine after the injection of histamine,[1] the first possibility was assumed to be more likely. Since it was, therefore, of interest to investigate whether the derivative is formed through interaction of histamine with the intestinal wall or with intestinal contents, the intestines were removed from a freshly killed dog and the contents were removed from segments of colon and ileum which were then washed clean and tied. The loops were filled with 10 ml of saline containing 1 mg of histamine per ml. To the intestinal contents 1 mg histamine per gram was added after removal of 1 g samples. All preparations were incubated at 37°C and 1 ml or 1 g samples were withdrawn after appropriate intervals. Each sample was diluted to 20 ml with water. The mixture was shaken and centrifuged and chromatograms were prepared from 1 ml of the supernatant by diluting to 5 ml, adding salt mixture, ether and butanol extraction and cotton succinate purification as described in the previous experiments. The chromatograms illustrated in Fig. 6 show that the histamine derivative was formed rapidly in the contents taken from the colon. but not in the washed loops in which histamine disappeared without formation of acetyl histamine. In the contents taken from the ileum traces of the histamine derivative were formed.

Approximate Rf values (Fig. 6)

Control acetyl histamine		Histamine derivative	
Strip 9	.74	Strip 7	.75
		” 8	.75

Formation of the histamine derivative after addition of histamine to human feces. The preceding experiment suggested intestinal contents as a site for the conversion of histamine. It was, therefore, of interest to investigate if human feces could convert histamine in a similar manner. Freshly passed human feces were divided into 2 samples. To one sample 1 mg/g histamine was added and 1 g portions were taken immediately from the control and histamine-containing feces. Both samples were then incubated and 1 g portions were withdrawn at convenient intervals. Chromatograms were prepared as described in the previous experiment and are shown in Fig. 7. Strip 1 (normal stool) shows a small amount of the histamine derivative present before histamine addition. Strip 2 represents 10 µg acetyl histamine and 10 µg histamine added to normal stool as a control and the following strips represent stool samples taken at various times after the histamine addition. It is of interest to note the appearance of an unidentified band (Rf 0.81) after 30 and 55 hours incubation. Strip 10 represents normal stool after 24 hours incubation.

Approximate Rf values (Fig. 7)

Control acetyl histamine		Histamine derivative	
Strip 2	.72	Strip 3	.71
		” 4	.70
		” 5	.70
		” 6	.70
		” 7	.71
		” 8	.72
		” 9	.72

The influence of autoclaving human feces on the formation of the histamine derivative. The previous experiment showed that histamine, when added to fresh human feces, is converted effectively to acetyl histamine. In order to test whether the reaction is due to a biological process or due to a chemical reaction not requiring living matter, a freshly passed human stool specimen was divided into 2 portions, one of which was autoclaved at 200 lb pressure for 20 minutes. After taking 1 g samples from the normal and autoclaved portions, 1 mg histamine per gram of stool was of histamine. Since the amount of conjugate present was small, the size and purification of the samples had to be modified: to 20 ml of urine were added 6 g of the salt mixture described. The samples were extracted twice with 10 ml of butanol. The combined butanol extracts were aerated until free from ammonia and were then passed through cotton succinate.[4] After passage of the butanol extracts, the cotton succinate was washed with 2 ml of acetone which was followed by elution with 2 ml of 0.5 N H_2SO_4, followed by 2 ml H_2O. The combined H_2SO_4-H_2O eluate was neutralized with 10 per cent NaOH and 1.5 g of the salt mixture of Na_3PO_4 and Na_2SO_4 was added. The resulting solution was extracted with 2 ml of butanol, which was then applied to the paper strips as in the previous experiment. Control strips were prepared as in the foregoing experiment. The amounts of histamine derivative appearing on the chromatograms are much smaller than those observed in the previous experiment. This is partly explained by the smaller amount of histamine employed and partly by the failure of cotton succinate to absorb acetyl histamine quantitatively.[2] Moreover, Anrep has reported that man, after the oral administration of histamine, does not excrete as much of the histamine derivative in the urine as does the dog.[1] It is of interest to note that free histamine, as well as histamine derivative, appears in the human urine early after the histamine administration (Strip 2, Fig. 3).

Approximate Rf values (Fig. 3)

Histamine		Control acetyl histamine		Histamine derivative	
Strip 3 (control)	.53	Strip 3	.69	Strip 2	.69
Strip 2	.53			Strip 4	.68
				Strip 5	.68
				Strip 6	.70

Hydrolysis of acetyl histamine to histamine. The foregoing experiments provide evidence that the substance excreted in the urine of dog and man after histamine ingestion is acetyl histamine. In order to obtain evidence for the identity of the compound described by Anrep[1] with acetyl histamine, a sample of synthetic acetyl histamine was hydrolized under the conditions used by Anrep for the hydrolysis of the urinary compound to histamine. Six mg of acetyl histamine were dissolved in 5 ml of water and 1 ml concentrated hydrochloric acid. 0.01 ml (10 μg) of the solution was applied to paper strips both before and after boiling the solution for one and one-half hours. A control strip was prepared by applying 10 μg of histamine. Chromatograms were prepared as in the first experiment, and are shown in Fig. 4. It is evident that acetyl histamine is easily converted to histamine under the conditions employed. A trace of acetyl histamine was still present after the hydrolysis, possibly because of the relatively large amount of acetyl histamine employed. The approximate Rf values of pure histamine (Strip 3) is 0.55 and of histamine obtained from the hydrolysis of acetyl histamine (Strip 2) 0.55.

Excretion of the histamine derivative in human feces after oral administration of histamine. The previous experiments presented evidence for the identity of the urinary histamine derivative with acetyl histamine. Because of the possibility that the histamine derivative might also appear in the feces, as well as the urine after feeding histamine, 3 stool samples were collected before, 6 and 20 hours after the administration of the histamine to the human subject. To 1 g (wet weight) of each sample was added 10 ml water. A fourth sample was prepared by adding 20 μg of each histamine and acetyl histamine to 1 g of the stool sample collected before the histamine administration. All samples were shaken, filtered and 3 g of the previously mentioned salt mixture was added to the filtrates which then were twice extracted with 10 ml portions of ether. This was followed by extraction with 2 ml of butanol which were passed through cotton succinate. The elution of the cotton succinate and the preparation of the chromatogram shown in Fig. 5 were carried out as in the third experiment. Strip 1 (normal stool) shows no band. Strip 2, representing the stool sample obtained 6 hours after the ingestion of histamine, shows a pronounced acetyl histamine band below and a weak histamine band above. Strip 3, corresponding to the stool sample taken 20 hours after the ingestion of histamine, shows a weaker acetyl histamine band and only a very

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Chemistry of the Liver Cytoplasm of Normal, Fasted and Cirrhotic Mice.

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An impairment of the mechanism for the mobilization of lipide material from the liver has been reported to be present in both starvation[1] and cirrhosis.[2] MacLachlan[1] has observed that the liver of fasted albino male mice accumulates neutral fat in such amounts, that in the first 2 days, the quantity of total lipide increased 2 to 3 fold. As the starvation is continued, the amount of total lipide decreases even below that of the normal, and the phospholipides show a downward trend during this entire period of starvation. Wright[2] observed that the alcoholic cirrhotic liver is pale, enlarged, and most of the liver cells are distended with fat. This lipide may result from the inadequate diet observed in many cirrhotics. The liver cell has a cytoplasm which appears to be quite labile,[3] so that an investigation of the chemical changes occurring in this portion of the cell, during cirrhosis and fasting, might give some indication as to the integral processes involved.

Three experimental groups of animals were studied: 1. Normal, fully fed animals. 2. Animals which were fasted for 24 hours, and 3. Cirrhotic animals which were fasted for 24 hours.

Materials and methods. Male mice, 5 to 7 months of age, of the C_3H strain were utilized in this experiment. Hepatic cirrhosis was produced by feeding 0.1 ml of 40% carbon tetrachloride in olive oil to mice every 4 days for a period of 200 days.[4,5] Cirrhosis was confirmed by histologic examination.[†]

Livers, of mice anaesthetized with ether, were removed after clamping the portal vein and cutting the hepatic vein. The blood that was present in the liver was thus allowed to drain.

The cytoplasmic extract was prepared essentially according to the method of Claude.[6] Livers from 10 mice were pooled for each group, ground in a mortar, suspended in 4 volumes of alkaline saline (2 ml 0.1 N NaOH per liter of physiologic saline), then spun at 1400 × g in the Sorvall angle centrifuge. The decanted supernatant fluid was considered to contain a major aliquot of the total cytoplasm. Lipides that came to the surface in the various preparations were resuspended in the extract before the analyses were performed. It was assumed that all of the lipide that rose to the surface belonged to the cytoplasm, since Rosenfeld[3] observed that an increase of lipide during various pathologic conditions, *e.g.* phosphorus poisoning, was purely cytoplasmic in nature.

Separate aliquots were removed from the cytoplasmic extract for the determination of total nitrogen,[7] phosphorus,[8] "ribonucleic acid," and lipide. Total solids were obtained by heating an aliquot of the cytoplasmic extract in a tared weighing bottle over a boiling water bath until the fluid from the extract disappeared. The bottles were then placed in an oven at 105°C for one hour.

The "ribonucleic acid" was determined essentially by Brown's modification[9] of the

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[1] MacLachlan, P. L., Hodge, H. C., Bloor, W. R., Welch, E. A., Truax, F. L., and Taylor, J. D., *J. Biol. Chem.*, 1942, **143**, 473.

[2] Wright, A. W., *Arch. Path.*, 1941, **32**, 670.

[3] Rosenfeld, G., *Ergebn. d. Physiol.*, 1903, **2**, 50.

[4] Edwards, J. E., *J. Nat. Canc. Inst.*, 1941, **2**, 197.

[5] Eschenbrenner, A. B., and Miller, E., *J. Nat. Canc. Inst.*, 1945, **3**, 251.

[†] The authors are indebted to Dr. R. A. Huseby for the pathologic examination.

[6] Claude, A., *J. Exp. Med.*, 146, **84**, 51.

[7] Ma, T. S., and Zuzaga, I., *Ind. Eng. Chem.*, Anal. Ed., 1942, **139**, 395.

[8] Fiske, T. S., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

added to each portion. One gram samples were withdrawn immediately after mixing the histamine into the stool and again after 4 hours incubation. The chromatograms shown in Fig. 8 were prepared as described above. The autoclaved sample, before the addition of histamine, shows a faint band, due to the histamine derivative which does not increase in intensity after incubation with histamine (Strips 1, 2, 3). The bands due to the histamine derivative increase markedly in color intensity on incubation of the non-autoclaved sample after addition of histamine (Strips 4, 5, 6). Pure acetyl histamine was applied to Strip 7 as a control.

Approximate Rf values (Fig. 8)

Control acetyl histamine		Histamine derivative	
Strip 7	.71	Strip 1	.73
		,, 2	.73
		,, 3	.73
		,, 4	.72
		,, 5	.71
		,, 6	.71

Attempts to convert histamine to acetyl histamine with pure cultures of E. coli and Aerobacter aerogenes. The experiment showing the formation of the histamine derivative made it appear likely that conversion of the histamine was due to fecal organisms. Histamine, was, therefore, added to a pure culture of *E. coli* and of *Aerobacter aerogenes* incubated in a simple glucose-salt medium.[6] Samples of the cultures were withdrawn at intervals and chromatograms, shown in Fig. 9, were prepared in a manner similar to that described in the previous experiments. Both organisms appear to form acetyl histamine in small quantities from added histamine. In addition, the organisms apparently are able to produce other unidentified substances from histamine which give colored bands with the color reagent employed.

Approximate Rf values (Fig. 9)

Control acetyl histamine		Acetyl histamine formed in culture	
Strip 3	.70	Strip 2	.71
,, 6	.71	,, 4	.70

Discussion. The experiments presented provide evidence that the histamine derivative recoverable from urine and feces of dog and man is acetyl histamine.* This substance is probably identical with the histamine derivative described by Anrep.[1] The fact that acetyl histamine is formed from histamine in normal but not in autoclaved feces and the ability of common fecal organisms to form traces of acetyl histamine in pure culture suggests that the observed acetylation of histamine in feces may be due to bacteria. The fact that *E. Coli* and *Aerobacter aerogenes* in pure culture are not able to produce acetyl histamine as effectively in quantities comparable to those obtained from feces may be due to several obvious differences in the conditions obtaining in stool samples and in simple culture media. Other fecal organisms may be able to acetylate histamine much more effectively. The possibility that histamine may also be acetylated elsewhere in the body, *e.g.* the liver, has been suggested and is not excluded by the present experiments. It should be noted that on occasion small quantities of acetyl histamine and histamine were observed in normal stool and urine samples.

Conclusions. 1. The observation that a histamine derivative appears in the urine of dog and man after oral histamine administration is confirmed.

2. The histamine derivative can be effectively separated from histamine by paper chromatography.

3. The histamine derivative is identical with acetyl histamine as shown by paper chromatography.

4. Acetyl histamine is readily formed from histamine added to feces of dog and man.

5. It is suggested that histamine is acetylated in the intestinal contents to acetyl histamine which is partially absorbed from the intestinal tract and excreted in the urine as well as in the feces.

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[6] Kohn, H. I., and Harris, J. S., *J. Pharm. and Exp. Therap.*, 1941, **73**, 343.

* Dr. Tabor and Dr. Rosenthal have isolated and crystallized the urinary histamine derivative. The melting point and chemical analysis characterize the compound as acetyl histamine. (Private communication).

gen content has entered the lipide extract. Cirrhosis affects the lipide content so that it increases above normal but not as high as the normal fasted liver cytoplasm. The phospholipide in the cirrhotic liver decreases, but here also there seems to be a lipide present with a high nitrogen content.

The iodine value of both the normal fasted and the cirrhotic-fasted liver cytoplasms decreased below that of normal. These results are in accord with those of Winter[12] in that he found that after feeding carbon tetrachloride to rats the iodine value of the liver fatty acids decreased from 111 to 100.

Summary and discussion. Chemical changes in the cytoplasm of the mouse liver cell after a short fast and at cirrhosis are reported. These changes occur most dramatically in the lipide fraction during a 24 hour fast resulting in an increase of 3 to 4 fold above normal. This lipide is non-phospholipide in character. Fasted cirrhotic liver differs from the fasted normal liver cytoplasm in that the lipide does not increase so markedly and the phospholipide decreases to about 60% of the normal fasted value. The iodine values of the normal-fasted and the cirrhotic-fasted liver cytoplasm fatty acids decrease below that of the normal values.

In addition to a lipide increase in the fasted-cirrhotic liver cytoplasm there is also a decrease of "ribonucleic acid" and phospholipide in one gram wet weight of the ground fasted cirrhotic liver.

It is possible that there is an interchange of phospholipide during a fast as evidenced by the lipide nitrogen to phosphorus ratio. This interchange would consist of substituting normal phospholipide with a lipide or phospholipide which contains a great deal of nitrogen.

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[12] Winter, J. C., *J. Biol. Chem.*, 1939, **128**, 283.

16857 P

Electrical Alternation in Experimental Coronary Artery Occlusion.

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The purpose of this preliminary note is to report a high incidence of electrical alternation in experimental coronary artery occlusion.

Methods. In open-chested nembutalized dogs,[1] the left anterior descending coronary artery was occluded for periods of 30 seconds to 32 minutes. Electrocardiographic tracings were made with the exploring electrodes in the cavity of the left ventricle and on the epicardial surface or precordium. Thirty-three experiments were performed on 11 dogs.

Results. Electrical alternation occurred in 8 of 9 dogs (89%) which developed electrical signs of myocardial ischemia[2] following coronary artery occlusion. One dog died of ventricular fibrillation, and one dog failed to develop any electrical signs of ischemia following occlusion for 32 minutes. Electrical alternation occurred about 2 minutes after occlusion, was variable in duration (several seconds to 20 minutes), and disappeared either spontaneously or within 3 seconds to 5 minutes after release of the constriction. Alternation of the ST-T and QRS complexes, alone or together, was observed, the most common being of the ST segment (Fig. 1). There was alternation in the extent of ST elevation in the epicardial leads, and of ST segment elevation or depres-

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* Dazian Fellow in Medicine.

[1] Hellerstein, H. K., and Katz, L. N., *Am. Heart J.*, 1948, 30, 184.

[2] Bayley, R. H., and LaDue, J. S., *Am. Heart J.*, 1944, 28, 54.

TABLE I.
Chemistry of the Liver Cytoplasm of Normal, Fasted, and Fasted-Cirrhotic Mice.*

	Mg per g wet weight liver					
Experiment†	Total solids	N	P	"RNA"	N/P	N/"RNA"
Fully fed	206	22.2	2.54	11.2	8.75	1.98
	235	29.1	2.68	12.1	10.9	2.41
Fasted	294	24.1	2.47	11.3	9.78	2.13
	377	30.5	3.09	14.2	9.88	2.15
Fasted	213	23.4	2.28	7.26	10.2	3.22
Cirrhotic	241	27.0	2.46	8.18	10.9	3.31

* It was assumed that nearly all of the cells were ruptured[13] and the concentration of the chemical constituents, *e.g.*, N, P, "RNA", total lipide, and phospholipide in the cytoplasmic extract, was the same as their concentration in the original ground tissue. With this assumption it is possible to consider the chemistry on a wet weight basis and thus avoid the discrepancy that may arise in dry weight due to the large amounts of lipide material present.

† Each figure results from the analysis of one pool of 10 animals.

TABLE II.
Lipide Chemistry of the Liver Cytoplasm of Normal, Fasted, and Fasted-Cirrhotic Mice.

	Mg per g wet weight liver			
Experiment†	Total lipide	Phospholipide	N/P (atomic)	Iodine value
Fully fed	43.4	22.0	1.10	125
	46.5	22.2	1.24	129
Fasted	140	23.9	1.41	92.5
	177	25.2	1.48	95.5
Fasted	67.5	16.1	1.50	90.2
Cirrhotic	81.5	17.4	1.64	91.8

* Each figure results from the analysis of one pool of 10 animals.

orcinol-HCl method in which the pentose values are corrected for the presence of hexose. The "ribonucleic acid" was calculated by multiplying the total tetranucleotide ribose by the factor 2.14.

Lipide was extracted with Bloor's solution (alcohol:ether; 3:1). Bloor's solution was re-extracted with petroleum ether, care was taken to wash the lipide extract with water to remove any of the non-protein nitrogen contaminants.[10] A weight of lipide was obtained by evaporating the petroleum ether under reduced pressure from tared 10 ml volumetric flasks. The iodine value was determined by the method of Kretchmer *et al.*[11]

Experimental results. Table I shows the actual amount of nitrogen, phosphorus, and "ribonucleic acid" present in the liver cytoplasm obtained from one gram wet weight of liver. There are no differences between the various liver cytoplasms as concerns nitrogen, but the phosphorus appears to have decreased in the fasted-cirrhotic liver which is probably in part due to the decrease observed in the "ribonucleic acid" in these livers. This decrease in "ribonucleic acid" is obviously not due to the fast but to the cirrhosis since there is no decrease in "ribonucleic acid" in the fasted livers.

Total lipide (Table II) increases approximately 3 to 4 fold after a 24 hour fast, but this excess lipide seems to be entirely non-phospholipide in character since the phospholipide remains constant. The lipide phosphorus to nitrogen ratio indicates that the phospholipide present in the cytoplasm has been altered in that a lipide of a higher nitro-

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[9] Brown, A. H., *Arch. Biochem.*, 1946, **11**, 269.

[10] Folch, J., and Van Slyke, D. D., *J. Biol. Chem.*, 1939, **129**, 539.

[11] Kretchmer, N., Holman, R. T., and Burr, G. O., *Arch. Biochem.*, 1946, **10**, 101.

[13] Barnum, C. P., and Huseby, R. A., *Arch. Biochem.*, 1948, **19**, 17.

sion in intracavitary leads. Alternation of the QRS complex showed variation of less than 10% in QRS areas between large and small beats. Areas of the ST-T complexes varied on an average of 40% between beats.

Discussion. Fundamentally, the factor underlying all forms of alternation is a marked prolongation of the refractory phase of some part of the heart leading to alternating localized block.[3,4] In Wiggers' laboratory, Orias[5] and Tennant[6] showed that masses of cardiac fibers are deleted from contracting as a result of ischemia following coronary artery occlusion. Green[7] demonstrated that this deletion may occur alternately, *viz.*, mechanical alternation occurred after experimental coronary artery occlusion with the area of ischemia bulging alternately, and producing an alternation of aortic pressure. Similarly, we may assume that electrical alternation of injury effects seen in our experiments is on the same basis, *i.e.*, a failure of certain fractions of ischemic myocardium to respond on alternate beats.

Alternation was predominantly of the ST-T complex, probably because injury currents (of rest and of activity)[1] persist longer than the transient initial changes of depolarization and repolarization in early acute myocardial ischemia.[2] Alternation in the direction of the T wave implies alternation in the epicardial-endocardial order of repolarization, while alternation of the magnitude of the T wave implies alternation of the rate or intensity but not of the direction of repolarization.[8]

Previous temporary occlusions predisposed the myocardium to the production of alternation. The etiological significance of ventricular premature beats remains unsettled since alternation occurred with or without preceding ventricular premature beats. Heart rate was not significantly increased, so that electrical alternation cannot be explained on this basis.[9]

Summary. Electrical alternation developed in 8 of 9 dogs (89%) which survived coronary artery occlusion and showed electrocardiographic signs of myocardial ischemia. Electrical alternation occurred within 2 to 3 minutes after occlusion and was transient. Repeated temporary occlusions predisposed to the development of electrical alternation.

Alternation was predominantly of the ST-T complex, although less marked alternation of the QRS complex and of the T wave also occurred.

It is postulated that in our experiments, electrical alternation of injury effects is due to the failure of certain fractions of ischemic myocardium to respond on alternate beats.

The authors express their appreciation to Dr. Harold Feil for his helpful criticism and guidance.

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[3] Katz, L. N., Electrocardiography, Lea and Febiger, Phil., 2nd ed., 1948, 808.

[4] Katz, L. N., and Feil, H. S., *Am. J. M. Sc.*, 1937, **194**, 601.

[5] Orias, O., *Am. J. Physiol.*, 1936, **114**, 407.

[6] Tennant, R., and Wiggers, C. J., *Am. J. Physiol.*, 1935, **112**, 351.

[7] Green, H. D., *Am. J. Physiol.*, 1936, **114**, 407.

[8] Hellerstein, H. K., and Liebow, I. M., *Am. Heart J.*, in press.

[9] Lewis, T., The Mechanism and Graphic Registration of the Heart Beat. Shaw and Sons, 3rd ed., London, 1925, 436.

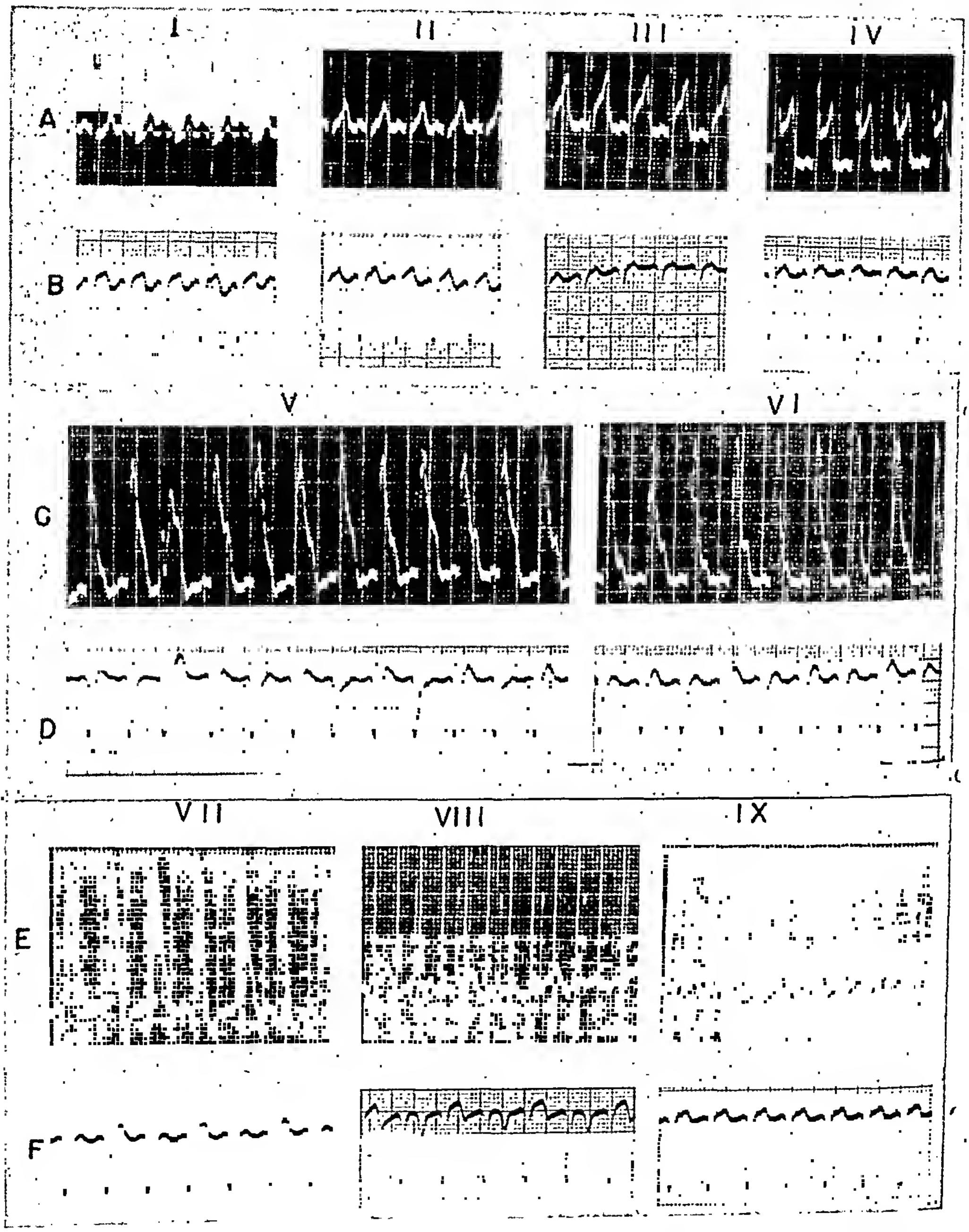
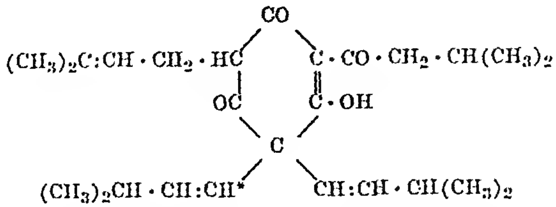


FIG. 1.

Electrical alternation following coronary artery ligation. Rows A, C, and E are epicardial records, and B, D, and F intracavitary records from left ventricle. Column 1 is control. Columns 2, 3, 4, 5, 6, 7, and 8 are 25, 55, 105, 243, 253, and 293 seconds after occlusion of left anterior descending artery. Note T wave changes precede ST deviation. Alternation is more marked after appearance of a premature ventricular beat (Column 5). ST-T alternation in Columns 5, 6, and 8. T wave alternates *in direction* in cavity lead in Column 8. Column 9 shows recovery 7 minutes after release of occlusion.



lupulon, made preliminary tests of the effect of this agent on experimental tuberculosis infections desirable.

Toxicity of lupulon. The toxicity of lupulon on single and repeated intramuscular injections was determined in inbred white mice. Crystals of lupulon were dissolved (1.5%) in cottonseed oil. The acute LD_{50} was found to be 600 mg per kilo. Deaths occurred within 1 to 12 hours. At autopsy, it was noted that incomplete absorption of the solution had occurred even after 5 days. This condition was observed in animals given only half of the LD_{50}. Daily injection of 60 mg per kilo over 4 weeks was tolerated without gross evidence of harmful effects. Histopathologic examination of tissue from these animals revealed small areas of leukocytic infiltration of the liver as well as foci of degeneration in the renal tubules.

Oral toxicity of lupulon, 5% suspended in gum acacia solution (6% in water) was determined in mice. Intragastrically, 1500 mg per kilo killed half of the animals within one hour of administration. All treated mice were depressed in contrast to those animals given the drug intramuscularly. Immediately before death, mice given doses twice the LD_{50} were excited and exhibited convulsions of a tetanic character. Congested and hemorrhagic lungs were observed at autopsy.

Antituberculous activity. Six samples of crystalline lupulon have been tested *in vitro* against tubercle bacilli (H37Rv) in Dubos' fluid medium.[15] Serial dilutions were made by pipetting appropriate amounts of lupulon solution in propylene glycol into 5 ml of the medium. Early samples were used in 1% solution in propylene glycol with the aid of heat, and later samples were dissolved first in 95% ethyl alcohol up to 10% solution and then diluted to 0.1% in propylene glycol. Growth of mycobacteria was inhibited at a dilution of 1:40,000 whether heat or ethyl alcohol was used to facilitate solution of the lupulon in the glycol. Humulon had a much lower potency and was not investigated further.

Various wetting agents,‡ including "Tween 60," "G-2144," "Nopacol," "Neutronyx," and "Triton," have been used to substitute for "Tween 80" in Dubos' medium without any effect on the tuberculostatic activity of lupulon.

The activity of lupulon has also been tested in serial dilution in the presence of a number of other antituberculous substances such as promin, streptomycin, subtilin, and 2,3-dimercaptopropanol (BAL). Sub-bacteriostatic concentrations of the latter agents (*e.g.*, 1:800, 1:3,200,000, 1:400,000 and 1:100,000, respectively) were used in the Dubos medium. Lupulon checked the growth of tubercle bacilli at 1:40,000 despite the presence of any of these agents. No synergism or antagonism occurred. The lack of synergism between lupulon and BAL is in contrast with the potentiation of action of subtilin and streptomycin by BAL, previously reported.[3]

Groups of 20 mice were infected intravenously with 0.02 mg of the H37Rv strain of *M. tuberculosis* grown in Dubos' medium. Lupulon was administered by two different routes. In one group of infected animals, it was given intramuscularly as a 1.5% solution in cottonseed oil at a daily single dose of 60 mg per kilo. In another group, it was administered intragastrically as a 3% suspension in 6% gum acacia solution (aqueous)

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[15] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, 83, 409.

‡ Kindly supplied by Dr. R. J. Dubos, Rockefeller Institute for Medical Research, New York. Mention of this and other products does not imply that they are endorsed or recommended over others of a similar nature not mentioned.

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Antituberculous Activity and Toxicity of Lupulon for the Mouse.*

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Wong and others have reported from the University of California on the *in vitro* activity of subtilin against *Mycobacterium tuberculosis*.[1-4] The parenteral application of this antibiotic has been interfered with by its relative insolubility in physiologic saline.[5] Antibiotics characterized by lipid solubility might overcome this difficulty. Two such agents prepared at the Western Regional Research Laboratory are lupulon and humulon derived from hops (*Humulus lupulus, L.*). Lupulon has the structure given below:[6] In humulon the side chain marked "*" is replaced by a hydroxyl group. A method for the isolation of lupulon from hops by direct crystallization was discovered by Michener *et al.*[7] and further simplified by Alderton.[8] The initial crystallization occurred in a vacuum concentrate of a direct petroleum ether extract of hops.

The antibacterial properties of hops have been recognized for many years, in connection with their use in brewing. Walker and Parker[9] reported lupulon and humulon to be 29,000 and 4,000 times, respectively, as active as phenol in restricting acid production by *Lactobacillus bulgaricus*. Shimwell[10] noted that hop extracts inhibited a considerable number of saprophytic Gram-positive bacteria, but not Gram-negative bacteria. Acid-fast mycobacteria and other pathogens were not tested. Michener *et al.*[7] reported antifungal activity for lupulon and humulon. Subsequent to the earlier observations reported in this paper, Reynolds[11] found inhibition of acid-fast organisms at 1:100,000 for lupulon and at 1:10,000 or 1:20,000 for humulon when this agent was incorporated in agar by a streak technic. Salle[12] obtained similar results against *M. tuberculosis* tested on both Long's and Dubos' media.

Hops have long been regarded as having hypnotic properties. Steidle[13] reported that hops produce paralysis and decreased excitability of the striated muscle and of motor nerve endings in frogs. Sikorski and Rusiecki[14] reported lupulon and humulon to be sedative for pigeons and small birds and somewhat less active in mice. These indications of pharmacologic action after oral administration, together with the fat-soluble nature of

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* University studies were supported in part by a grant from Eli Lilly and Company, Indianapolis 6, Indiana.

† Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[1] Wong, S. C., Hambly, A. S., Jr., and Anderson, H. H., *J. Lab. Clin. Med.*, 1947, **32**, 837.

[2] Anderson, H. H., and Wong, S. C., *Tuberculology*, 1946, **8**, 77.

[3] Anderson, H. H., and Chin, Y. C., *Science*, 1947, **106**, 643.

[4] Chin, Y. C., *Fed. Proc.*, 1947, **6**, 317; Chin, Y. C., and Anderson, H. H., unpublished results.

[5] Wilson, R. H., Lewis, J. C., and Humphreys, E. M., *Fed. Proc.*, 1948, **7**, 266.

[6] Richter, V. von, Organic Chemistry, or, The Chemistry of the Carbon Compounds, Vol. II, pp. 400-401, Nordemann Publ. Co., New York, 1939.

[7] Michener, H. D., Snell, N., and Jansen, E. F., *Arch. Biochem.*, 1948, **19**, 199.

[8] Alderton, G., unpublished results.

[9] Walker, T. K., and Parker, A., *J. Inst. Brewing*, 1937, **43**, 17. CA 31:1152.

[10] Shimwell, J. L., *J. Inst. Brewing*, 1937, **34**, 111, 191.

[11] Reynolds, D. M., in preparation for publication.

[12] Salle, A. J., private communication.

[13] Steidle, H., *Arch. Exp. Path. Pharmacol.*, 1931, **161**, 154.

[14] Sikorski, H., and Rusiecki, W., *Bull. intern. acad. polon. sci., Classe Med.*, 1936, **73**, 83. CA 32:9280.

TABLE II.
Relative Abundance of Mycobacteria* in Lesions of Treated and Untreated Mice.

Antibiotic	*Experiment*	*Route*	*Lungs*	*Spleen*	*Liver*	*Kidneys*	*Heart*	*Sum total*
Controls	I	—	471	19.3	68.3	70.7	83.8	713
,,	II	—	353	277	278	170	436	1514
Lupulon	II	Oral	78	27	27	80	170	382
,,	I	I.M.	121	4.5	2.0	93.4	10.1	231
,,	II	I.M.	14.6	3.8	12.9	2.7	334	368

* See Table I for key..

of tissue reaction, as measured by the development of lesions, in all but the renal and cardiac tissues. The latter organs in all groups did not differ appreciably in occurrence of lesions. In the lungs, not only was there a lower percentage of tuberculous tissue in lupulon-treated mice than in the controls but the type of tissue reaction was also different. The lesions in the lungs in the lupulon-treated mice were predominantly proliferative, while those in the controls were predominantly necrotic and exudative.

Discussion. An over-all examination of these data showed a significantly lower number of mycobacteria in lesions of lupulon-treated mice. Thus far, in chemotherapeutic trials in animals, only indefinite numbers of mycobacteria in lesions have been reported.[16-18] In our opinion, the numbers of mycobacteria in the lesions is of great importance to the solution of the problem of ultimate control. The use of a drug should be for a relatively short period in terms of the life span of the individual. On cessation of therapy, there should be left in lesions a small enough number of bacilli for the natural defense mechanism of the host to combat successfully.

Since the lipid fraction of tubercle bacilli is a major part of the organism, it would appear reasonable to expect a fat-soluble antibiotic to have a marked affinity for these bacteria, and that this affinity might be reflected in the animal. Fat-soluble usnic acid, which has a relatively low tuberculostatic effect *in vitro*, has a definite effect in guinea pigs.[19] This hypothesis is also suggested by our observations; *e.g.*, the lipid soluble lupulon is active *in vivo*.

Lupulon, like the aerosporins and polymyxins, appears to have an affinity for the renal tubules when it is given intramuscularly. Whether these changes in the tubules are reversible or not remains for further experiment. The relatively mild leukocytic infiltration of the liver may not be significantly harmful. This may indicate a mobilization of the antibiotic from the site of injection and its storage in the liver. Storage in the liver would be compatible with the finding that the greatest reduction in mycobacteria occurred, among lupulon-treated animals, in this organ.

Summary. Lupulon, a fat-soluble antibiotic derived from hops, has relatively low *in vitro* activity (1:40,000) as compared with other antituberculous agents. Despite this observation, lupulon (given orally or intramuscularly) *was active* against experimental mouse infections of *M. tuberculosis*. Following intramuscular administration, significantly lower numbers of acid-fast organisms occurred in lesions of treated animals. The approximate numbers relative to the control were: In the liver, 34 to 1; heart, 8 to 1; spleen, 4 to 1; and lungs, 4 to 1; but not in the kidneys. In orally treated animals the ratios were: Liver, 10 to 1; heart, 3 to 1; spleen, 10 to 1; lungs, 5 to 1, and kidneys, 2 to 1. The over-all difference was a reduction of approximately 4 to 1 by either route of administration.

Lupulon given intramuscularly within its effective range produced some foci of degen-

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[16] Pierce, C., Dubos, R. J., and Middlebrook, G., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 173.

[17] Youmans, G. P., Raleigh, G. W., and Youmans, A. S., *J. Bact.*, 1947, 54, 409.

[18] Levaditi, C., and Vaisman, A., *Comp. rend. Soc. Biol.*, 1948, 142, 43; *ibid.*, 1948, 142, 308.

[19] Marshak, A., *Public Health Reports*, 1947, 62, 3.

TABLE I.
Tuberculostatic Activity of Lupulon in Infected Mice.

Antibiotic	Experiment	Route	Approx. LD$_{50}$ mg/kg	Daily dose mg/kg over 30 days	Local effects noted grossly	Avg gross lung lesions*	Tissue reaction in all organs examined	Relative abundance of T.B. in all organs†	No. animals in each group
Controls	I	—	—	—	—	3.7	18	713	20
,,	II	—	—	—	—	5.0	24	1,514	20
Lupulon	II	Oral	1500	150 (twice daily)	None	2.3	18	382	20
,,	I	I.M.	600	60 (daily)	,,	1.9	12	231	20
,,	II	I.M.	600	60 (daily)	,,	2.8	16	368	20

* *Tissue Reaction:* Number indicates total (average) degree of lesions (larger means greater involvement); *e.g.*, Lung: 8 = 50-100% of tuberculous tissue in the whole organ as observed grossly as well as microscopically; 4 = 25-50%; 2 = 10-25%; 1 = 1-10%; 0 = no gross lesions. Spleen and liver: 8 = 10 lesions per low power field; 4 = 5-10 lesions; 2 = 2-5; 1 = lesions in any portion; 0 = no apparent lesions. Kidney and heart: Number = No. of lesions in the section; exception 4 = 4 or more.

† *Abundance of Mycobacteria:* Number indicates total (average) relative number of mycobacteria in all lesions; *e.g.*, 1,000 = more than 10 bacilli to each cell; 100 = 1-10 to each cell; 10 = 1 bacillus to 1-10 cells; 1 = 1 bacillus to 1-5 lesions; 0 = no organisms.

at a dose of 150 mg per kilo at 12-hour intervals. Other infected animals served as untreated controls. The results are shown in Table I. The evaluation of the gross tuberculous lesions in the organs was based on histopathologic sections stained with hematoxylin and eosin for lesions and by acid-fast methods for determining abundance of tubercle bacilli present.

Despite its relatively low *in vitro* activity, lupulon exerted considerable suppressive effect on the development of tuberculosis in mice, whether it was given intramuscularly or orally. It appeared to check the multiplication of tubercle bacilli to a similar extent when given orally. The latter method gave greater suppression in the development of tuberculosis in the second series of experiments where a difference in multiplication of mycobacteria was apparent.

Table II gives further details regarding the relative abundance of mycobacteria in lesions of treated and untreated mice. In comparing the prevalence of microorganisms in the lungs, spleen, liver, kidneys, and heart of the control animals, notable differences were apparent at the end of the 30-day period with their relative abundance in lupulon-treated animals. The ratios (in Experiment I) approximated from 34 to 1 (comparison of controls with lupulon-injected mice) for liver, 8 to 1 for heart, 4 to 1 for spleen, and 4 to 1 for lungs. After oral use (in Experiment II) the ratios were: Liver 10 to 1, heart 3 to 1, spleen 10 to 1, lungs 5 to 1, kidneys 2 to 1: an over-all 4 to 1 difference. Only the renal lesions had a slight and perhaps insignificantly greater number of mycobacteria in lupulon-injected animals, in the first but not in the second series. Renal damage might be related to tubular changes which followed continual administration of this antibiotic, and these may have been accentuated by the extent of renal infection, with concurrent drug damage to this tissue. Another possibility is that the foci of degeneration noted in the renal tubules (in infected intramuscularly treated animals) may have permitted more extensive tuberculous involvement.

The lupulon-treated mice had lower indices assay for vitamin A.

Experimental. d,l,α-tocopherol acetate. Rats of the Sprague-Dawley strain were given a stock diet until they weighed 40-50 g. Half of them were then transferred to a diet deficient in both vitamins A and E and having the following percentual composition: Vitamin-free casein, 20%; glucose, 67.5%; salt mixture,[13] 2.5%; ground cellophane, 3%; lard, 2%; yeast, 5%; viosterol (in peanut oil), 40 drops per kg. The other half were given the same diet except that hydrogenated vegetable fat (Spry) replaced the lard and 12.5 μg of *d,l,α*-tocopherol acetate[*] were added per kg of diet. Based on food consumption each animal received 70-80 γ of α-tocopherol acetate and 4-5 γ of tocopherols daily. In a second series the amount of α-tocopherol acetate added to the diet was 100 mg per kg. giving a daily intake of about 600 γ.

At the end of the deprivation period of 3-4 weeks when xerophthalmia or decline in weight or both became evident, the animals were properly grouped and vitamin A containing oils were administered daily, orally, by syringe with bent blunt needle. The Standard U.S.P. Reference Oil No. 2 and the several shark liver oils[†] to be tested were diluted with cottonseed oil[‡] to contain 2 I.U. of vitamin A per 0.1 cc (Reference Oil) or per 0.05 cc (liver oils). The oils were kept in the cold and new dilutions were made up fresh each week.

In Table I are the average figures obtained from these two series of animals. They indicate surprising and certainly acceptable uniformity in the times required for the development and cure of the symptoms of deficiency, whether α-tocopherol acetate was present in the diet or not. The weight gains on the reference oil were only half as great as those on the test oils, and the cure of xerophthalmia was also slower. This was doubtless due to the fact that this oil, as was reported later,[14] contained 30% less vitamin A than indicated. This figure was subsequently verified by the Carr-Price method against a standard vitamin A capsule kindly supplied by Distillation Products, Inc.

The vitamin A potency of the fish oils as indicated by the anti-xerophthalmic data agreed well with that of the chemical assays provided (Carr-Price, Rosenthal, Beckman). With the figures for gain in weight, the agreement was unsatisfactory, largely because of the unsuspected inferiority of the reference oil, and the consequent lack of a comparable standard dose-level.

d,l,α-tocopherol. The tests with the alcohol, made some time after those just described, differed from the earlier ones in several minor respects. Drisdol was used in place of viosterol as a source of vitamin D. Neither α-tocopherol nor vegetable oil was added to the basal diet of the experimental animals; α-tocopherol[§] was given to them by mouth during the assay period only and in the same diluting olive oil which also contained the 2 I.U. of the vitamin A. The control animals received their vitamin A in olive oil. The sources of vitamin A were limited to the two reference oils U.S.P. Nos. 2 and 3 (the former after several years' storage in a dark ice-box). Finally, in lieu of a chemical determination of the liver stores of vitamin A, which at the moment could not be made on the necessary micro-scale, the survival time was determined after the close of the assay eration in renal tubules. Such pathologic changes were not observed in infected animals given this antibiotic orally in effective doses. The single LD_{50} on intramuscular administration was 600 mg per kilo in mice; on oral application, 1,500 mg per kilo.

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[13] Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

[*] Generously supplied by Hoffman-La Roche, Inc.

[†] We are indebted to Dr. J. Murray Luck for samples of these oils and the results of chemical assays on them, on the basis of which they were diluted for the bio-assays.

[‡] Cottonseed oil was used designedly, if not wisely, to stabilize the vitamin A in the oils before absorption. During the assay period, therefore, the vitamin E deficient animals on the test and reference oils received daily 20 and 40 γ of tocopherols, respectively; those on the low level of dietary α-tocopherol acetate had a 20-50% increase in the potential vitamin E intake, those on the high level, 3-7% increase. The data indicate that cottonseed oil was inconsequential in its effect on the final results and that it may have accomplished its purpose.

[14] Collison, E. C., and Orent-Keiles, E., *Ind. Eng. Chem.*, Anal Ed., 1945, **17**, 378.

[§] Kindly supplied by Merck and Company.

16859

Tocopherol vs. Tocopherol Acetate as a "Sparer" of Vitamin A.

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The early observation[1] that materials containing vitamin E delayed the auto-oxidative destruction of carotene and vitamin A in fatty food mixtures was explained when the tocopherols were shown to be antioxidants.[2] That such protection may augment and conserve the vitamin A stores of an animal was first demonstrated by Moore[3] and has been confirmed repeatedly, as detailed in several reviews and some recent reports.[4]

The evidence suggests that inhibited oxidation in or near the alimentary tract[5] accounts for the survival of carotene and vitamin A. Recently,[6] this protective action appeared to be confined to preserving the store of vitamin A already deposited in the liver. The various forms of vitamin A behave differently. The results are modified by the form and method of administering the preparations of vitamin A[7] and vitamin E[8]. Other compounds having possible stabilizing effects[9] may be present and effective.

Several reports have stated or implied that the stabilizing action of the tocopherols is not limited to the alimentary tract but extends to the tissues of rats.[8,10] This was not demonstrable in rabbits.[11] Some information on this question might emerge from experiments with the esters or α-tocopherol, among them the acetate, which is also an oil, and is a dependable source of vitamin E, perhaps because it is not auto-oxidizable and has no antioxygenic action. Presumably, α-tocopherol acetate is hydrolyzed in the intestinal tract, and the free alcohol should therefore be available in the tissues in quantities undiminished by having provided prior stabilization in the food.

The only pertinent observation on the acetate appears to be that of Bacharach,[12] to the effect that only at high levels of feeding and for an extended period (60 days) was any conservation of vitamin A demonstrable. A comparison of α-tocopherol and its acetate was therefore undertaken, to assess the relative protective value of each by simple experiments patterned after the U.S.P. biotration; in the case of lard, this is between 0.05 and 0.10% of α-tocopherol.[15] Very recently, in similar experiments,[16] there is a suggestion that 0.5 mg α-tocopherol is an optimum dosage for the storage of vitamin A in the liver; the effect on vitamin A storage in the kidney was irregular. The liver stores of vitamin A from administered β-carotene were diminished by the larger doses of α-tocopherol and kidney storage was again irregular.

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[1] Mattill, H. A., *J. Am. Med. Assn.*, 1927, **89**, 1505.

[2] Olcott, H. S., and Emerson, O. H., *J. Am. Chem. Soc.*, 1937, **59**, 1008.

[3] Moore, T., *Biochem. J.*, 1940, **34**, 1321.

[4] Hickman, K., *Ann. Rev. Biochem.*, 1943. **12**, 353; Moore, T., *Vitamins and Hormones*, 1945, **3**, 1; Mattill, H. A., *Ann. Rev. Biochem.*, 1947, **16**, 177; McCoord, A. B., *et al.*, Food Technol., 1947, **1**, 263; Foy, J. R., and Morgareidge, K., *Analyt. Chem.*, 1948, **20**, 304.

[5] Hickman, K., *et al.*, *J. Biol. Chem.*, 1944, **152**, 303, 313, 321.

[6] Popper, H., Steigmann, F., and Dyniewicz, H. A., *Gastroenterology*, 1948, **10**, 987.

[7] Halpern, G. R., and Biely, J., *J. Biol. Chem.*, 1948, **174**, 817.

[8] Lemley, J. M., *et al.*, *J. Nutrition*, 1947, **34**, 205.

[9] Sherman, W. C., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 207; Esh, G. C., and Sutton, T. S., *J. Nutrition*, 1948, **36**, 391.

[10] Davies, A. W., and Moore, T., *Nature*, 1941, **147**, 794; Hove, E. L., and Harris, P. L., *J. Nutrition*, 1946, **31**, 699; Lundberg, W. O., *et al.*, *J. Biol. Chem.*, 1947, **168**, 379.

[11] Major, R., and Watts, B. M., *J. Nutrition*, 1948, **35**, 103.

[12] Bacharach, A. L., *Quart. J. Pharm. Pharm.*, 1940, **13**, 138.

Unfortunately, no indication was secured as to the vitamin A stores of our animals on α-tocopherol acetate, following the assay period. The larger of the two dosages, 0.6 mg per day, even though it was within the optimum range and extended over both depletion and assay periods, may have been insufficient; it can hardly have been excessive. In any case, there was no response. The stabilization which the acetate can provide for vitamin A in the alimentary tract is limited to the amount of α-tocopherol available from it in the interval between hydrolysis and absorption. Perhaps, as with triglycerides, complete hydrolysis of the ester is not necessary for absorption, in which case any protective action in the tissues would depend upon the amount absorbed and the rate of subsequent hydrolysis. Information is needed on the rate of hydrolysis and absorption of tocopherol esters. The possible antioxygenic role of tocopherols in the tissues remains to be clarified.

Summary. 1. In bio-assays of vitamin A, α-tocopherol, but not α-tocopherol acetate, increased the rate of gain in the assay period. In a post-assay depletion period, animals that received α-tocopherol during the assay period survived 30-80% longer than controls.

2. As judged by gain in weight in the assay period, there is an optimum dosage of α-tocopherol; animals receiving 1.5 mg per day gained less than those receiving 0.5 mg. This conforms to the antioxygenic behavior of α-tocopherol.

3. Some of the possible causes for the failure of α-tocopherol acetate to conserve vitamin A are briefly discussed.

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[15] Golumbic, Calvin, *Oil and Soap*, 1943, **20**, 105.

[16] Johnson, R. M., and Baumann, C. A., *J. Biol. Chem.*, 1948, **175**, 811.

16860 P

On the Specificity and Differentiation of Cholinesterases.

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Cholinesterases (ChE) of different sources may be separated into two types, the "s" and the "e."[1-3] The behavior of the two enzymes toward noncholine esters has been supposed to be the major difference between them. It has been assumed that the e-type could catalyze only the hydrolysis of certain choline esters;[4] therefore it has been also called "specific" or "true" cholinesterase.

The venoms of the colubrid snakes can split acetylcholine[5] and some noncholine esters.[6] In a recent investigation, it was shown that

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[*] On leave from the University of Basel, Switzerland.

[1] Richter, Derek, and Croft, Phyllis G., *Biochem. J.*, 1942, **36**, 746.

[2] Mendel, B., and Rudney, H., *Biochem. J.*, 1943, **37**, 59.

[3] Zeller, E. A., and Bissegger, Alfred, *Helvet. chim. acta*, 1943, **26**, 1619.

[4] Mendel, B., Mundell, Dorothy B., and Rudney, H., *Biochem. J.*, 1943, **37**, 473.

[5] Zeller, E. A., Enzymes of Snake Venoms and Their Biological Significance. In: *Advances in Enzymology and Related Subjects of Biochemistry* (edited by F. F. Nord), New York, Interscience Publishers, Inc., 1948, **8**, 459.

[6] Bovet Nitti, F., *Experientia*, 1947, **3**, 283.

TABLE I.
Influence of α-Tocopherol Acetate on Bio-assay of Vit. A.
+E animals had 12.5 mg *d,l,α*-tocopherol acetate/kg of diet.

Source of curative Vit. A		Avg body wts at depletion, g	Avg gain in assay period, g	Xerophthalmia	
				Appeared in depletion period at avg days	Cured in assay period at avg days
Refs. Oil 2	—E	84 (6)*	28 (1)†	23	22
Refs. Oil 2	+E	79 (6)	27 (1)	22	20
Oil 3	—E	74 (5)	58 (2)	23	21
Oil 3	+E	80 (8)	52	23	16
Oil 4	—E	80 (8)	46	24	18
Oil 4	+E	80 (7)	46	23	16
Oil 5	—E	80 (8)	47	23	19
Oil 5	+E	82 (7)	44 (1)	23	16
+E animals had 100 mg *d,l,α*-Tocopherol acetate/kg of diet.					
Refs. Oil 2	—E	76 (6)	25 (1)	28	17
Refs. Oil 2	+E	78 (6)	33 (1)	22	18
Oil 7	—E	76 (8)	65	23	11
Oil 7	+E	74 (7)	54	22	15
Oil 11	—E	75 (5)	50 (3)	25	10
Oil 11	+E	76 (7)	54	23	11
Oil 14c	—E	72 (6)	57 (1)	25	10
Oil 14c	+E	72 (7)	53 (1)	22	12

* No. of animals.
† No. of animals which died during the period and not included in the average.

TABLE II.
Influence of α-Tocopherol on Bio-assay of Vitamin A.

Reference oil and amt. of tocopherol, (mg)		Avg body wt at depletion, g	Gain in assay period, g	Xerophthalmia		Avg survival in post-assay depletion, days
				Appeared in depletion period at avg days	Cured in assay period at avg days	
2	.0	85 (8)*	3	29	13	11
2	.5	91 (6)	17 (2)†	31	12	19
2	1.5	91 (6)	11 (2)	29	13	19
3	.0	90 (5)	4 (4)	30	16	14
3	.5	80 (8)	25	28	15	19
3	1.5	87 (7)	13 (2)	34	10	19
2	.0	80 (7)	11 (1)	27	11	17
2	.3	85 (5)	24 (3)	28	6	24
3	.0	82 (8)	24	27	7	15
3	.7	78 (7)	38 (1)	27	8	28

* No. of animals.
† No. of animals that died during the assay period and not included in the average.

period when the administration of supplements ceased. Two series of observations were made with each of the reference oils; in the first, 0.5 and 1.5 mg of α-tocopherol were administered with each oil, in the second 0.3 mg with oil No. 2, and 0.7 mg with oil No. 3.

Despite the unexplained high mortality in some of the lots, the data in Table II show that (1) survival in the post-assay depletion period was prolonged by α-tocopherol given in the assay period; (2) there was an optimum α-tocopherol dosage, perhaps 0.5-0.7 mg daily, beyond which the effectiveness of the vitamin A dosage decreased, as indicated by gains in weight in the assay period. Both of these findings are confirmatory of earlier reports[5] and point to an antioxygenic rather than a biological function of α-tocopherol. The stabilization of fats by α-tocopherol *in vitro* also demonstrates an optimum concen-

TABLE II.
Enzymatic Hydrolysis of β-Chloroethyl Acetate and Acetylcholine.
Same method as described in Fig. 1; same enzyme preparations as in Table I. Final concentration of acetylcholine: 0.0067 M; final concentration of β-chloroethyl acetate; 0.033 M for the first three and 0.016 M for the last preparation. Q_{ACh} resp. $Q\beta_{ClA}$ give the number of microliter of CO_2 produced in 1 hour, calculated from reaction of zero order.

Source of cholinesterase	Type	Q_{ACh}	$Q\beta_{ClA}$
Erythrocytes (human) purified	e	669	249
Snake venom (Naia melanoleuca)	e	540	129
Plasma (human)	s	405	39
Parotid (guinea pig)	s	711	72

split this ester (Table II).

Detailed information about methods and enzyme and substrate preparations will be given in an extensive publication.

Conclusions. The ability to hydrolyze noncholine esters can no longer be used as a criterion to distinguish the two cholinesterase types, since both groups of enzymes are able to split noncholine esters; for example, ethyl chloroacetate. Another noncholine ester, β-chloroethyl acetate, is even preferentially attacked by the e-type ("true" or "specific" ChE).

The use of eserine to decide whether a hydrolysis is catalyzed by a cholinesterase or another esterase is of limited value, because the s-type ("pseudo" or "unspecific" ChE) is inhibited by this substance only in the presence of acetylcholine, but *not in that of* ethyl chloroacetate. The e-type is inhibited in both cases.

Ethyl chloroacetate plus eserine and β-chloroethyl acetate can be used as new means of distinction of the two groups of cholinesterases.

16861 P

Variations in the Vitamin B_{12} Content of Selected Samples of Pork and Beef Muscle.*

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In a recent paper[1] a fairly quantitative assay procedure for a growth factor in liver preparations was described. This assay procedure involves placing rats on the basal ration for a 2 week depletion period and following the growth response during another 2 week period when the material to be tested is given. Further work has shown that crystalline vitamin B_{12} will give a quantitative response in this assay (Fig. 1).

Henderson *et al.*[2] have reported wide variations in the ability of pork muscle, when fed to female rats as the source of protein, to produce normal lactation. Less than one-third of the young were reared in certain experiments; however, in other experiments the lactation was comparable to that obtained with rations containing beef. Beef-fed rats

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[1] Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, in press.

[2] Henderson, L. M., Schweigert, B. S., Mozingo, A. K., and Elvehjem, C. A., *J. Nutrition*, 1948, 36, 479.

several noncholine esters—for example, ethyl chloroacetate (ECIA)—are hydrolyzed at a high rate by many of the colubrid venoms, and that this hydrolysis is due to cholinesterase.[7] Since the colubrid cholinesterase belongs to the e-type,[8] the question arose as to whether other e-cholinesterases also have the ability to split EC1A.

In order to solve the problem of whether other cholinesterases display a pattern of specificity similar to that of the snake venom enzyme, experiments were carried out with the e-cholinesterase of the erythrocytes of human beings. The hemolyzed erythrocytes and a purified preparation derived therefrom[2] catalyzed the hydrolysis of EC1A at an easily measurable rate. With the use of mixtures of acetylcholine and EC1A, it could be shown that with the concentration of acetylcholine varying from 0.004 M to 0.025 M, and with the concentration of EC1A varying from 0.035 M to 0.075 M, the resulting hydrolysis rate ($Q_{ACh} + Q_{EC1A}$). One typical example is given in Fig. 1.

TABLE I.
Inhibition of the Enzymatic Hydrolysis of EC1A by Eserine.

Source of cholinesterase	Type	Inhibition, %
Erythrocytes (human) purified*	e	84-94
Snake venom (Naia melanoleuca)†	e	100
Plasma (human)‡	s	4-11
Parotid (guinea pig)§	s	13-

Same method as described in Fig. 1.

* 0.2 ml of erythrocyte-ChE, prepared according to the method of Mendel and co-worker.

† 40 μg of dried venom of Naia melanoleuca.[10]

‡ 0.083 ml of heparinized plasma of human beings.

§ 0.5 cc of extract from homogenized parotids from guinea pigs, prepared with 10 volumes of saline solution. Final concentration of eserine: 0.2-0.3 10^{-3}; final concentration of EC1A 0.016-0.05 M.

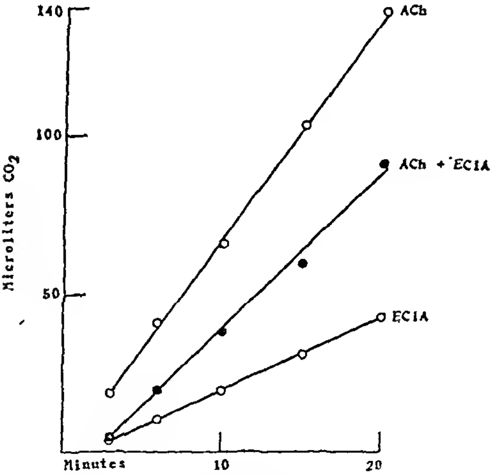

FIG. 1.
Hydrolysis of ethyl chloroacetate and acetylcholine by hemolyzed erythrocytes. Manometric measurement of the CO_2 produced from bicarbonate by the liberation of acids.[9] Erythrocytes were washed three times with saline, hemolyzed with an equal volume of distilled water and diluted with two volumes of bicarbonate-*Ringer* ("Ringer-30"). Three tenths milliliter of this preparation was used in a total volume of 3 ml. Final concentration of acetylcholine: 0.0067 M; final concentration of EC1A: 0.075 M. Blanks resulting from the enzyme solutions and the substrates were subtracted.

The enzymatic hydrolysis of EC1A in the presence of snake venoms[10] and of purified ChE from human erythrocytes[2] is inhibited by caffeine and other hydroxylpurines and by eserine (Table I) and prostigmine in the same way, and to the same extent, as has been shown to be true of acetylcholine.[3,11]

Serum from human beings and extracts from guinea pig parotid glands, typical sources of the s-ChE ("unspecific," "pseudo" ChE), split EC1A very rapidly. This hydrolysis is only slightly inhibited by eserine (Table I).

Another striking difference between the two esterases was found when β-chloroethyl acetate was used. This noncholine ester is easily attacked by purified erythrocytes and snake venom. Experiments carried out with mixed substrates and with eserine gave the same results as with EC1A, indicating that this substance is also hydrolyzed by e-ChE. Human serum is comparatively less able to

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[7] Zeller, E. A., *Helvet. physiol. et pharmacol. acta*, 1948, **6**, C36.

[8] Zeller, E. A., *Experientia*, 1947, **3**, 375.

[9] Ammon, R., *Arch. f. d. ges. Physiol.*, 1933, **233**, 486.

[10] Zeller, E. A., and Utz, D. C., *Helvet. chim. acta*, in press.

[11] Nachmansohn, D., and Schneemann, H., *J. Biol. Chem.*, 1945, **159**, 239.

16862

Influence of Adrenergic Blocking Drug [N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr] On Pyrogenic Reaction.[*]

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An important mechanism of the febrile response to pyrogen is vasoconstriction[1-3] with reduction of skin temperature.[2,4-10] The site of action of pyrogen in the production of fever is central rather than peripheral since such fever is prevented by certain lesions of the central nervous system.[11-13] Pyrogen induced vasoconstriction and fever are mediated in part *via* the sympathetic nervous system, partial or complete removal of which modifies or prevents such vasoconstriction[2,5] and delays and reduces fever.[14] Evidence is here presented that N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr,[†] a potent adrenergic blocking agent,[15-17] reduces the febrile response of the dog to pyrogenic material.

Five dogs received intravenous injections of 50 mcg/kg of "Pyromen,"[‡] a purified pyrogenic material obtained from pseudomonas aeruginosa. Six dogs were injected intravenously with 3 mg/kg of N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr 30 minutes prior to the injection of pyrogen. Five days later the order was reversed and dogs previously pre-treated with blocking agent now served as their own controls and vice versa.

Rectal temperatures were determined at 30 minute intervals and data from the experiments on these 11 dogs are given in Table I. The first value is the average temperature during the 6 hour period following injection of pyrogen, and the second is the maximum temperature recorded during the pyrogenic reaction. Both are expressed as increments over basal temperature. Paired comparisons show the mean responses of the two experimental periods to be significantly different. The reduction in average response accomplished by the blocking agent is of the order of 25%.

These studies imply only slight participation of the sympathetic nervous system in the pyrogenic reaction. However, while the dose of N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr is sufficient to cause complete reversal of the blood pressure response to injected epinephrine in the dog, its potency in preventing sympathetic nerve mediated

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[*] Aided by a grant from Baxter Laboratories, Inc., Morton Grove, Ill.

[1] Fremont-Smith, F., Morrison, R. L., and Makepeace, A. W., *J. Clin. Invest.*, 1929, **7**, 489.

[2] Perera, G. A., *Arch. Int. Med.*, 1941, **68**, 241.

[3] Altschule, M. D., Freedberg, A. S., and McManus, M. J., *J. Clin. Invest.*, 1945, **24**, 878.

[4] Ranson, S. W., Jr., *Arch. Int. Med.*, 1938, **61**, 285.

[5] Pinkston, J. O., *Am. J. Physiol.*, 1934, **110**, 448.

[6] Brown, G. E., Allen, E. V., and Mahorner, H. R., Thromboangiitis Obliterans, W. B. Saunders and Co., Philadelphia, 1928.

[7] Brown, G. E., *Surg., Gyn., Obs.*, 1934, **58**, 297.

[8] Friedlander, M., Silbert, S., and Bierman, W., *Am. J. Med. Sci.*, 1940, **199**, 657.

[9] Rosenthal, W., *Arch. f. Physiol.-Physiol. Abth.*, 1893, 217.

[10] Hildebrandt, F., *Arch. f. exp. Path. u. Pharm.*, 1943, **201**, 278.

[11] Jona, J. L., *J. Hyg.*, 1916, **15**, 169.

[12] Ranson, S. W., Jr., Clark, G., and Magoun, H., *J. Lab. Clin. Med.*, 1939, **25**, 160.

[13] Chambers, W. W., and Windle, W. F., *Fed. Proc.*, 1947, **6**, 89.

[14] Pinkston, J. O., *Am. J. Physiol.*, 1935, **111**, 539.

[†] Supplied by Parke, Davis and Co., through the courtesy of Dr. George Rieveschl, Jr.

[15] Loew, E. R., and Micetich, A., *J. Pharm. Exp. Therap.*, 1948, **93**, 434.

[16] Wells, J. A., and Rall, D. P., *Fed. Proc.*, 1948, **7**, 264.

[17] De Vleeschhouwer, G. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 298.

[‡] Supplied by Baxter Laboratories, Inc., through the courtesy of Dr. N. M. Nesset.

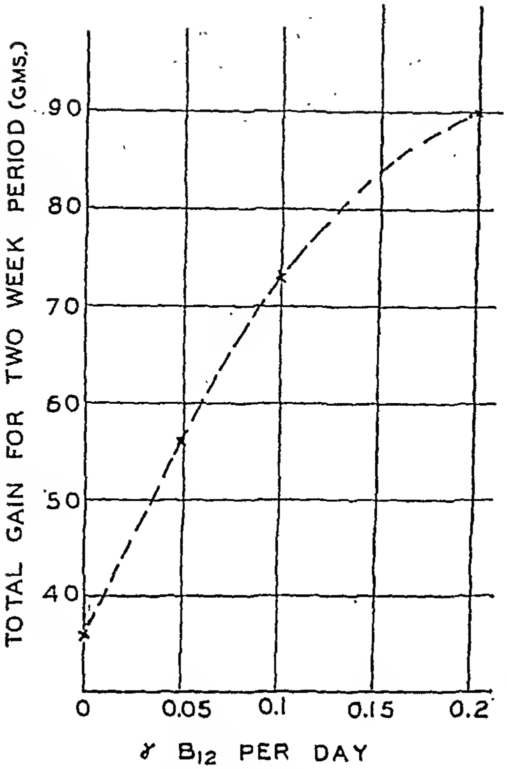

Fig. 1.

Response of rats receiving graded levels of B_{12}.

usually reared 80 to 90% of the young. The wide variations encountered when pork was fed as the source of protein led to the testing of samples of beef and pork by this new assay method for B_{12} activity.

A commercial liver preparation, assayed for vitamin B_{12} content by means of rats using crystalline vitamin B_{12} as a standard, was used as a reference. One USP antipernicious anemia unit of liver preparation was found to contain approximately one microgram of vitamin B_{12}.

These results show that the beef samples contain twice as much B_{12} as the normal pork sample while the abnormal pork sample contains only a trace or no B_{12}. The results given for the meat samples are minimum values since they are compared with B_{12} which was injected intraperitoneally. The wide variation in the B_{12} content of pork samples is significant in that it may offer an explanation for the variations in the ability of pork samples to produce normal lactation in female rats reported by Henderson and coworkers.[2]

The variable results with pork are not surprising since swine, being monogastric animals, may not be supplied with ample quantities of B_{12} and possibly other factors which may be produced by the microorganisms in the rumen of cattle.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamins including B_{12}, and to Dr. B. L. Hutchings of the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid.

Daily supplement	Total avg gain (g) two wk	Min. B_{12} (γ) per 100 g of sample
None	36	—
5 g beef, round steak (Sample 1)	74	2
5 g beef, round steak (Sample 2)	74	2
5 g pork, shoulder (normal)*	56	1
5 g pork, shoulder (abnormal)†	39	Trace
0.1 USP. unit Reticulogen (Lilly)‡	74	2000§

* From sow that lactated normally.
† From sow that showed abnormal lactation (young died after birth).
‡ Injected intraperitoneally.
§ Contains approximately 1 γ B_{12} per USP unit.

TABLE II.
Pyrogenic Reaction in 10 Curarized (Control) and 13 Curarized Dogs (Pre-treated) with Adrenergic Blocking Agent.

Latent period (min.)		Duration of rising temp. (min.)		Max. temp. post-inj. (°C)		Avg temp. during period of rising temp. (°C)		Avg temp. during 2 hr post-inj. (°C)	
Control	Pre-treated	Control	Pre-treated	Control	Pre-treated	Control	Pre-treated	Control	Pre-treated
20	90	110	230	1.61	1.72	1.01	.87	.73	.08
10	110	220	130	1.89	.89	1.11	.54	.52	.02
20	20	160	250	2.83	2.22	1.60	1.11	.86	.39
20	20	280	90	1.67	1.75	.98	1.16	.58	.93
20	30	110	10	1.61	.06	1.01	.05	.72	.04
20	180	150	10	1.39	.33	.79	.40	.44	.00
20	20	150	100	1.11	.25	.82	.18	.54	.14
20	20	180	30	1.61	.14	1.20	.14	.84	.07
20	30	110	120	1.22	.50	.73	.31	.52	.17
20	90	170	140	1.92	.86	1.42	.43	.88	.04
20		140		3.00		1.92		1.18	
30		160		1.64		1.19		.64	
20		190		1.97		1.23		.62	
Avg 20	61	163.9	111.0	1.805	.872	1.155	.519	.698	.188
Probability <0.01		0.10		<0.01		<0.01		<0.01	
Significance High		None		High		High		High	

All temperatures are rectal and are expressed as increments above basal temperature determined during pre-pyrogen control period.

group of animals pre-treated with blocking agent. The average response of the pre-treated group is 73% less than that of the control. On closer inspection of the individual dogs in the group of animals pre-treated with blocking agent, it is apparent that only 2 of them showed febrile reactions of any consequence and the reaction can be said to have been essentially abolished in the remainder.

It is concluded that a potent adrenergic blocking drug reduces the febrile response to the injection of pyrogenic material. In most curarized dogs the reaction is nearly abolished and greatly delayed in onset, but in two of them a nearly normal pyrogenic reaction was observed.

Assuming that the present drug alters the pyrogenic reaction because of its ability to block adrenergic nerves, then partial inhibition of the febrile response to pyrogen by this drug may be interpreted in several ways. The adrenergic blocking action of the drug may be only partial, or adrenergic nerve mediated vasoconstriction may contribute only partially to the pyrogenic reaction.

The febrile response in curarized dog is due in large part to vasoconstriction. Thus, partial reduction of the pyrogenic reaction under these conditions implies either incomplete adrenergic blocking action on the part of the drug or participation in the febrile response of a non-sympathomimetic vasoconstrictor substance.

In line with the latter interpretation are the observations that complete sympathectomy modifies but does not prevent the pyrogenic reaction in the cat[14] and that after complete sympathectomy and adrenal inactivation, the ears of some rabbits still showed delayed vasoconstriction associated with the rise in body temperature.[8]

Summary. A potent adrenergic blocking drug, N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr, has been shown to reduce and alter the febrile response of normal and curarized dogs to the intravenous injection of a purified pyrogenic material obtained from pseudomonas aeruginosa. This evidence is interpreted as supporting the hypothesis that the pyrogenic reaction is due, at least in part, to a reduction in heat loss caused by adrenergic nerve mediated vasoconstriction in the skin.

TABLE I.
Effect of Adrenergic Blocking Agent on Pyrogenic Response in Normal Dogs.

Dog No.	Avg temp. increment over 6 hr (°C)		Maximum increment in temp. (°C)	
	Control	Pre-treated	Control	Pre-treated
1	1.50	1.33	2.05	1.94
2	0.89	0.83	1.33	1.22
3	1.39	0.61	1.72	1.11
4	1.11	1.05	1.67	1.50
5	2.05	1.05	2.55	1.61
6	1.72	1.33	2.10	1.78
7	1.83	1.33	2.44	1.89
8	1.45	1.22	2.50	1.61
9	1.56	1.28	2.16	1.72
10	1.55	1.33	2.22	1.89
11	1.11	0.67	2.11	1.16
Avg	1.469	1.094	2.077	1.585
Probability	<0.01		<0.01	
Significance	High		High	

All temperatures are rectal and are expressed as increments over basal temperature determined during pre-pyrogen control period.

vasoconstriction in the skin is unknown.

In order to further test the role of the sympathetic nervous system in the pyrogenic reaction, the effect of the adrenergic blocking agent on the pyrogenic reaction was determined in curarized dogs. Under such conditions the major portion of the fever following injection of pyrogen is due to reduced heat loss.[18] Since the curarized dog cannot reduce heat loss by reduction of ventilation or reduction of radiating surface, it may be assumed that vasoconstriction is the mechanism of the fever.

Crystalline d-tubocurarine[§] was given intravenously in an initial dose of 1.5 mg/kg followed by hourly intramuscular injections of 0.5 mg/kg. Artificial respiration by means of a Starling-Palmer pump was adjusted to give a ventilation-oxygen consumption ratio of approximately 20. Rectal temperatures were obtained by means of a recording resistance thermometer. Room temperature was maintained at 27 ± 1°C.

Fifteen minutes after curarization 10 dogs were given 3 mg/kg of N-Ethyl-N-(2-bromoethyl)-1-naphthylenemethylamine · HBr by slow intravenous infusion. After a control period of approximately 1 hour these 10 dogs and 13 curarized control dogs were given 50 mcg/kg of pyrogen intravenously.

Data for the 13 control and 10 pre-treated animals are shown in Table II. Several values representative of the pyrogenic reaction are given. The first is the latent period or the period from injection of pyrogen to onset of fever. One effect of the adrenergic blocking agent appears to be significant prolongation of this latent period. The duration of the period of rising temperature is not significantly different in the two groups of dogs. The third value, the maximum increment in temperature during the pyrogenic reaction, is significantly lower in the group of dogs pretreated with blocking agent. The mean response for the group is 52% less than that of the control. The fourth value, the average increment in temperature during the period of rising temperature, is significantly lower in the group of animals pre-treated with the blocking agent. The mean response of the pre-treated group of animals is 55% less than that of the controls.

Thus the adrenergic blocking drug produces 2 alterations of the pyrogenic reaction in curarized dogs. It delays the onset of the response and reduces its magnitude. The last entry in Table II is a single figure which portrays both of these processes. This figure is the average increment in temperature during the 2 hour period following the injection of pyrogen, and it is significantly lower in the

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[18] Wells, J. A., and Rall, D. P., PROC. SOC. EXP. BIOL. AND MED., 1948, 68, 421.

[§] Supplied by Abbott Laboratories, through the courtesy of Dr. R. K. Richards.

TABLE I.
Summary of RBC Counts of Central and Peripheral Blood in Various Laboratory Animals.

Animal	Age	No. of counts ♂	No. of counts ♀	Avg RBC count of central blood, ×1000	Avg RBC count of peripheral blood, ×1000	Probability
Rat	1-99 Days	64	101	5,297	5,729	.1–.05
Mouse	Adult	15	15	9.648	10.571	.1–.05
Guinea pig	,,	12	8	6.422	6.160	.4–.5
Hamster	,,	5	8	8.283	9.388	.1–.05
Rabbit	,,	11	9	6.265	6.396	.6–.5
Dog	1 day	3	7	4.864	5.166	.6–.5
	Adult	6	14	6.284	6.529	.5–.4

heart, with about 15 minutes elapsing between samples. The order of sampling was reversed in about half the dogs in order to minimize the effect of splenic engorgement.

Peripheral and central red cell counts were also made in 10 newborn dogs at 1 day of age. No significant difference between central and peripheral red cell counts was found in young or adult dogs.

Discussion. In the study of central and peripheral red cell counts in 6 species, only the rat, mouse and hamster showed a difference between the two samples that approached significance. The statistical method used indicated, however, that the differences found in the averages could have been due to chance, since the probability factor was between 0.1 and 0.05, whereas a factor of 0.05 to 0.01 is necessary to demonstrate a high significance.

Summary. Red cell counts of blood secured from peripheral vessels were compared to counts from the heart or large vessels in the rat, mouse, hamster, rabbit, guinea pig, and dog. In none of these was a statistically significant difference found.

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[6] Carr, D. T., and Essex, H., *Am. J. Physiol.*, 1944, **142**, 40.

16864

Attempt at Percutaneous Introduction of d-Tubocurarine with a Direct Current into Muscles of Rabbits.*

E. E. GORDON† AND R. C. DARLING. (Introduced by R. F. Loeb.)

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Curare affords a possible indicator to gauge the depth of penetration of positive ions through the skin under the action of a galvanic current (electrophoresis, iontophoresis), since 1) its physiological action is solely on muscle and can easily be detected, 2) it is active in small amounts, 3) it is dissociated, at least in acidic solutions, into positive ions suitable for transfer at the positive pole. Previous work on the depth of penetration using dyes (Harpuder,[1] Harpuder and Rein[2]) have shown visible dye only in the epidermis in humans, and in dogs and rabbits only a faint coloration of the superficial muscle layers under extreme conditions. The possibility remains, however, that in deeper layers the dye was decolorized or otherwise made undetectable as rapidly as it entered them.

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* This research was aided by a grant to Columbia University by the Baruch Committee for Physical Medicine.

† Columbia Baruch Fellow in Physical Medicine.

[1] Harpuder, K., *Arch. Phys. Ther.*, 1937, **18**, 221.

[2] Rein, H., *Z. f. Biol.*, 1926, **84**, 41.

16863

Comparison of RBC Counts in Central and Peripheral Blood in Various Laboratory Animals.*

H. E. Ederstrom.† (Introduced by Benjamin De Boer.)

From the Department of Physiology and Pharmacology, University of Missouri School of Medicine, Columbia, Mo.

The observation that red cell counts of blood from the tail of the rat were higher than counts of blood from the larger vessels and heart was reported by Jolly.[1] Reichel and Monasterio[2] listed several authors who had found no difference between human arterial and venous red cell counts, nor between venous, ear, and finger samples. Haden and Neff[3] reported higher counts in peripheral than in longitudinal sinus blood of infants, but Lucas *et al.*[4] reported the opposite in the newborn.

Materials and methods. Two samples from each animal were taken with the same pipette, and each was counted in duplicate in a Neubauer-type hemacytometer. Hayem's solution was used as a diluting fluid. The significance of differences between central and peripheral counts was tested by Fisher's[5] "t" method of statistical treatment. All results are given in Table I.

Experiments and results. A total of 175 rats from 1 to 99 days of age was used. Tail vessels were dilated by warming at about 43°C for 30 seconds or less, and blood obtained by a transverse cut an inch or so from the distal end, following which a ligature was applied to stop bleeding. Central blood was procured by decapitation approximately one hour later. Averages of results indicate that peripheral blood had a higher red cell count than blood obtained by decapitation. However, statistical treatment indicates that this difference is not highly significant.

Thirty adult mice were sampled in the same way as rats. Warming the tail was omitted in 24 mice to determine whether this influenced red cell count, but no change was apparent. Tail and head samples did not differ significantly in red cell count in these animals.

Red cell counts in 13 Golden Syrian hamsters were made as described for mice. The difference between tail and head counts was found not statistically significant.

Twenty adult rabbits were anesthetized with 35 mg/kg sodium pentobarbital intravenously at least 30 minutes before sampling. Peripheral blood was obtained by nicking the ear margin, avoiding the larger vessels, and central blood secured about 20 minutes later by cardiac puncture. The order of sampling was reversed in half the animals. No significant difference between the red cell counts of the two samples was found.

The peripheral blood of 20 unanesthetized guinea pigs was obtained by nicking the ear after it had been warmed slightly under a lamp, and the central blood collected by cardiac puncture about an hour later. Red cell counts of ear and heart blood did not differ significantly.

Twenty adult dogs were anesthetized with 32.5 mg/kg sodium pentobarbital intraperitoneally at least 30 minutes before sampling. This interval permitted splenic engorgement, which influences blood concentration during this type of anesthesia, according to Carr and Essex.[6] Blood was taken from the tail and

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* This work was aided by a grant from the University of Missouri Research Council.

† Present address: St. Louis University School of Medicine, St. Louis, Mo.

[1] Jolly, M. J., *Compt. rend. soc. de biol.*, 1906, **60**, 564.

[2] Reichel, J., and Monasterio, G., *Klin. Wchnschr.*, 1929, **8**, 1712.

[3] Haden, R. L., and Neff, F. C., *Am. J. Dis. Child.*, 1924, **28**, 458.

[4] Lucas, W. P., Dearing, B. F., Hoobler, H. R., Cox, A., Jones, M. R., and Smyth, F. C., *Am. J. Dis. Child.*, 1921, **22**, 525.

[5] Fisher, R. A., Statistical Methods for Research Workers, 2nd edition, London, Oliver and Boyd, 1932, p. 151.

TABLE I.
Summary of RBC Counts of Central and Peripheral Blood in Various Laboratory Animals.

Animal	Age	No. of counts		Avg RBC count of central blood, ×1000	Avg RBC count of peripheral blood, ×1000	Probability
		♂	♀			
Rat	1-99 Days	64	101	5,297	5,729	.1–.05
Mouse	Adult	15	15	9,648	10,571	.1–.05
Guinea pig	,,	12	8	6,422	6,160	.4–.5
Hamster	,,	5	8	8,283	9,388	.1–.05
Rabbit	,,	11	9	6,265	6,396	.6–.5
Dog	1 day	3	7	4,864	5,166	.6–.5
	Adult	6	14	6,284	6,529	.5–.4

heart, with about 15 minutes elapsing between samples. The order of sampling was reversed in about half the dogs in order to minimize the effect of splenic engorgement.

Peripheral and central red cell counts were also made in 10 newborn dogs at 1 day of age. No significant difference between central and peripheral red cell counts was found in young or adult dogs.

Discussion. In the study of central and peripheral red cell counts in 6 species, only the rat, mouse and hamster showed a difference between the two samples that approached significance. The statistical method used indicated, however, that the differences found in the averages could have been due to chance, since the probability factor was between 0.1 and 0.05, whereas a factor of 0.05 to 0.01 is necessary to demonstrate a high significance.

Summary. Red cell counts of blood secured from peripheral vessels were compared to counts from the heart or large vessels in the rat, mouse, hamster, rabbit, guinea pig, and dog. In none of these was a statistically significant difference found.

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[6] Carr, D. T., and Essex, H., *Am. J. Physiol.*, 1944, **142**, 40.

16864

Attempt at Percutaneous Introduction of d-Tubocurarine with a Direct Current into Muscles of Rabbits.*

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From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Presbyterian Hospital, New York City.

Curare affords a possible indicator to gauge the depth of penetration of positive ions through the skin under the action of a galvanic current (electrophoresis, iontophoresis), since 1) its physiological action is solely on muscle and can easily be detected, 2) it is active in small amounts, 3) it is dissociated, at least in acidic solutions, into positive ions suitable for transfer at the positive pole. Previous work on the depth of penetration using dyes (Harpuder,[1] Harpuder and Rein[2]) have shown visible dye only in the epidermis in humans, and in dogs and rabbits only a faint coloration of the superficial muscle layers under extreme conditions. The possibility remains, however, that in deeper layers the dye was decolorized or otherwise made undetectable as rapidly as it entered them.

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* This research was aided by a grant to Columbia University by the Baruch Committee for Physical Medicine.

† Columbia Baruch Fellow in Physical Medicine.

[1] Harpuder, K., *Arch. Phys. Ther.*, 1937, **18**, 221.

[2] Rein, H., *Z. f. Biol.*, 1926, **81**, 41.

TABLE I.
Threshold Values* of Single and Tetanizing Shocks for Peroneal-Dorsi-Flexor System Before and Following Electrophoresis with *d*-Tubocurarine.

Control leg				Test leg				pH of solution	Remarks
Single stimulus		Tetanic stimuli		Single stimulus		Tetanic stimuli			
a	*p*	*a*	*p*	*a*	*p*	*a*	*p*		
15	15	11	12	12½	12½	10	8½	4	Control: wet dressings. with tubocurarine
12½	12½	8½	8	15	15	12	12½	4	
20	17½	15	15	12½	12½	12½	12½	4	Control: tubocurarine-free solution and electrophoresis
15	12½	10	10	8	7	6	7	4	
20	20	17½	16	17½	17½	15	15	7	
17½	20	15	16	22½	20	17½	15	7	
Avg (*p*—*a*) +0.4		Avg (*p*—*a*) 0		Avg (*p*—*a*) —0.6		Avg (*p*—*a*) —0.4			

* Threshold units are arbitrary, determined by ohmic resistance, and are proportional to intensity of stimulus.
a = before electrophoresis.
p = following electrophoresis.

Therefore, the present work using curare as a physiological rather than a histological indicator in the muscles is in order.

Nerve muscle preparations *in situ* were employed in rabbits anesthetized with sodium pentobarbital (30 mg/kg). The sciatic nerves were exposed bilaterally, the tibial nerves sectioned, and electrodes placed on each peroneal nerve. Upon electrical stimulation of the nerve, activity of the anterior compartment of muscles of the lower leg was set up to be recorded on a kymograph as dorsiflexion of the ankle joint. The upper legs were rigidly fixed by means of pins passed through the lower ends of the femora. Interference with circulation was avoided.

Electric contact of the positive electrode over the lower legs (hair clipped off) was by means of gauze pads soaked in a test solution and covered with a metal strip. The negative electrode was a large saline-soaked gauze pad placed over the abdomen (likewise with hair clipped off). The test solution containing d-tubocurarine‡ was made up to a concentration of 143 mg % in 50% ethyl alcohol containing 1:20,000 adrenalin. Alcohol was used because of the evidence that alcoholic solutions favor electrosmosis of positive ions through the skin,[3] and adrenalin because it was hoped that by sub-papillary vasoconstriction it would minimize removal of d-tubocurarine by the blood stream.[4] In 4 experiments the pH of this solution was approximately 4, and in 2 it was adjusted to approximately 7, to favor electroendosmosis through the epidermis.

The experimental plan was to utilize one leg as a test leg, the other as a control. Two types of control were employed. In the first type (4 observations) 15 ma. of direct current was passed through each leg for 20 minutes, but the solution over the control leg contained no d-tubocurarine, only alcohol, adrenalin and one drop of 0.85% NaCl. In the second type (2 observations) solutions containing d-tubocurarine were placed over both legs, but current was passed through only one. Thus, in the first type of control the only variable between the two legs was the presence of d-tubocurarine; in the second type the variable was the passage of current.

Using an electronic stimulator[5] which afforded graded intensity of stimuli of constant duration, the thresholds to a single shock and tetanizing stimuli (50 per second) were determined by recording the response on the kymograph immediately before the passage of the direct current through the legs and

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‡ We are indebted to the Abbott Laboratories, North Chicago, Ill., for a supply of this material.

[3] Rothman, S., *J. Lab. and Clin. Med.*, 1943, **28**, 1305.

[4] von Sallmann, L., *Arch. Ophthl.*, 1943, **29**, 711.

[5] Nastuk, W. L., and Borison, H. L., *Rev. of Scient. Instruments*, 1947, **18**, 669.

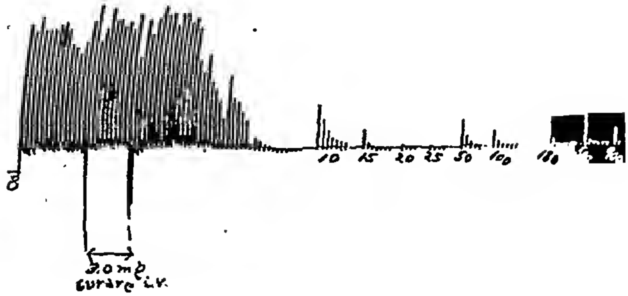

FIG. 1.
Effect of an intravenous curarizing dose upon threshold value of single electrical shocks in a nerve-muscle preparation.
Time scale = one stimulus per second. Regular stimulus at threshold (8 units) was begun, curare was injected and strength of stimulus increased as response fell (all indicated in subscript figure on tracing).

again immediately after the completion of flow of this current for a period of 20 minutes.

Threshold values to a single electric shock or tetanic stimuli remained unaltered in the control leg both after the simple application of d-tubocurarine wet dressings and electrophoresis with a d-tubocurarine-free solution. Similarly, the test leg showed no demonstrable change in thresholds following electrophoresis with the d-tubocurarine solution (Table I). Thus, within the limits of our method, such treatment does not introduce detectable amounts of the drug. It remains to measure the order of magnitude of tubocurarine in muscle that can be detected by our method.

A separate experiment using intravenous d-tubocurarine indicated that the presence of minute amounts of tubocurarine could be readily detected in the muscle. Fig. 1 illustrates the extreme degree of loss of excitability to electrical stimulation of our nerve-muscle system in a 3 kg rabbit receiving 3.0 mg of tubocurarine intravenously. Assuming a total muscle weight of 1.35 kg and 5 g as the weight of the muscles actually tested, then a maximum of 0.011 mg of tubocurarine reached the muscle. This value is an overestimation, since some of the drug injected must remain in the body fluids outside the muscles. Thus, our method is sensitive to less than 0.011 mg of tubocurarine.

Simple calculation (on the basis of electrochemical equivalents) shows that the passage of 15 ma for 20 minutes represents the ionic transfer of 0.1865 m.eq. of a monovalent positive ion or 58.2 mg in terms of tubocurarine.§ Since less than 0.011 mg (0.000035 m.eq.) of the drug can be detected by our method, to curarize our preparation by electrophoresis less than 1/5300 of the current need be transported by the tubocurarine ion, once it has crossed the skin barrier. Our results indicate that even such small amounts could not be introduced by the electric current. Thus, it appears that penetration of tubocurarine, to the muscle layer, if it occurs at all, yields concentrations too minute to be physiologically active.

Summary and conclusions. The question of the effective depth of percutaneous penetration of substances introduced by direct galvanic current was explored, utilizing the effect of d-tubocurarine upon muscle as an indicator system.

Observations on 6 rabbits indicated that threshold values to electrical stimulation of a suitable nerve muscle preparation was not altered after electrophoresis with d-tubocurarine.

Curarization of a rabbit with an intravenous dose revealed that to achieve an effect with tubocurarine electrophoresis only a maximum of 0.011 mg tubocurarine need reach the muscle.

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§ 1 m.eq. tubocurarine = 312 mg.

Thus, further evidence is adduced that penetration of substances through the skin by electrophoresis is limited.

16865

In vitro Effect of Certain Antibacterial Agents on Organisms Encountered in Bovine Mastitis.*

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In screening materials to learn which gave promise in the treatment of bovine mastitis, the *in vitro* effect of a number of antibiotic substances and sulfonamides was determined on some of the bacteria frequently associated with cases of bovine mastitis.

Some information is already available in the literature relative to the materials employed. Salle and Jann[1] reported that subtilin was bacteriostatic in high dilutions and bactericidal in lower dilutions for gram positive organisms, including *Mycobacterium tuberculosis.* Buggs and co-workers,[2] working with streptomycin *in vitro* found a great range in susceptibility of different strains of the same organism.

Goetchius and Lawrence[3] found that 3'-5' dibromo sulfanilanilide *in vitro* was effective in high dilution against *Streptococcus agalactiae, Streptococcus viridans,* and *Brucella abortus.* Schweinberg and Yetwin[4] stated that sulfamethazine *in vitro* was more bactericidal and bacteriostatic than sulfadiazine or sulfamerazine against *Eberthella typhosa, Escherichia coli,* and certain Salmonella. Francis *et al.*[5] reported that 4, 4' diaminodiphenyl sulfone (hereafter designated sulfone) showed promise against *Str. agalactiae* infections in the chick embryo and mouse. Burbaum and others[6] found that sulfadiazine was slightly inhibitory against *Corynebacterium diphtheriae.*

Methods. The organisms used in these experiments were *Str. agalactiae, Br. abortus, Pseudomonas aeruginosa, E. coli,* a coagulase-positive *Staphylococcus aureus,* and *Corynebacterium pyogenes. Salmonella typhimurium* was employed as a representative of the Salmonella.

A culture of each of the above organisms was first grown in tryptose broth for 24 hours at 37°C. Inocula for the actual tests were prepared from such broth cultures. The *C. pyogenes* broth culture was used undiluted while the others were diluted 1 to 100 in broth. Various concentrations of each of the antibacterial agents were prepared in 10 ml of sterile skimmed milk to which brom cresol purple was added as an indicator, and these tubes were inoculated with 0.2 ml of the suspensions of the organisms. Those cultures producing no visible change in milk were streaked on blood agar or nutrient agar slants to determine the extent to which the culture had grown in the presence of the agent. Thus the tests were carried out in skimmed milk

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station as a contribution from project number 475.

[1] Salle, A. J., and Jann, G. J., *J. Bact.*, 1946, **51**, 592.

[2] Buggs, C. W., Bronstein, B., Hirshfeld, J. W., and Pilling, M. A., *J.A.M.A.*, 1946, **130**, 64.

[3] Goetchius, G. R., and Lawrence, C. A., *J. Lab. and Clin. Med.*, 1946, **31**, 336.

[4] Schweinberg, F. B., and Yetwin, I. L., *J. Bact.*, 1946, **49**, 193.

[5] Francis, J., Peters, J. M., and Davies, O. L., *J. Comp. Path.*, 1947, **57**, 162.

[6] Burbaum, L., Nenner, N., and Dolgopol, V. B., *J. Bact.*, 1947, **53**, 507.

TABLE I.
Dilutions of Subtilin Necessary for Bactericidal and Bacteriostatic Action After 72 Hours Incubation.

Effect	*S. aureus*	*Str. agalactiae*	*C. pyogenes*
Bactericidal	1:80,000	1:80,000	1:100,000
Bacteriostatic	1:160,000	1:160,000	1:200,000

Subtilin lot 205-0, potency equivalent to 200, was obtained through the kindness of Dr. Hans Lineweaver, Western Regional Laboratory, Albany, Calif.

TABLE II.
Effect of Streptomycin on the Various Organisms After 72 hours Incubation, Reported as Units per ml.

Effect	*S. aureus*	*Str. agalactiae*	*C. pyogenes*	*S. typhimurium*	*E. coli*	*Br. abortus*	*Ps. aeruginosa*
Bactericidal	50	50	25	500	62.5	25	1000
Bacteriostatic	25	25	12.5	250	—	12.5	—

Streptomycin, lot No. 544, containing 573 units/mg, was obtained through the courtesy of Merck & Co., Rahway, N.J.

TABLE III.
Effect of Sulfonamides on the Organisms After 24 Hours Incubation.

Sulfonamide	Mg % conc.	*S. aureus*	*Str. agalactiae*	*C. pyogenes*	*S. typhimurium*	*E. coli*	*Br. abortus*	*Ps. aeruginosa*
Control		3+	3+	3+	3+	3+	3+	3+
3′-5′-dibromo-sulfanilanilide	8	2+	3+	3+	3+	3+	3+	3+
Sulfamethazine	8	3+	+	+	3+	—	—	3+
	1.6	3+	2+	+	3+	+	2+	3+
Sulfapyridine	8	2+	—	—	2+	±	+	±
	1.6	3+	+	3+	3+	+	+	2+
Sulfone	8	2+	±	—	3+	2+	+	3+
	1.6	3+	+	3+	3+	3+	2+	3+
Sulfadiazine	8	2+	±	—	2+	±	—	—
	1.6	3+	+	3+	3+	±	+	—
Succinyl sulfanilamide	8	3+	3+	3+	3+	3+	3+	3+
Sulfamerazine	8	2+	—	—	3+	—	—	±
	1.6	3+	±	—	3+	±	+	2+

— Complete inhibition of growth in milk, organisms not killed.
± Slight growth of organisms in milk.
+, 3+ Relative amounts of growth.

to provide somewhat the same medium in which the organisms are found growing in the bovine udder. The organisms were diluted to the approximate number likely to be found in mastitis.

Likewise, the various antibacterial agents were diluted to the approximate level which could be attained for a period of 10 to 12 hours after intramammary infusion in cows. The agents tested were subtilin, streptomycin, 3′-5′ dibromo sulfanilanilide, sulfamethazine, sulfapyridine, sulfone, sulfadiazine, succinyl sulfanilamide, sulfamerazine, disodium sulfone, and sodium sulfamethazine. The lowest concentration which prevented visible growth was termed the bacteriostatic concentration: that which killed all organisms, the bactericidal concentration.

Results. In Table I the results of studies on subtilin are compiled. *S. aureus* and *Str. agalactiae* were slightly more resistant than *C. pyogenes.* Table II shows the bacteriostatic and bactericidal concentration of streptomycin for the seven organisms. *Ps. aeruginosa* and *S. typhimurium* were much more resistant than the other organisms. In Table III the effects of various sulfonamides on the different organisms are shown. The 3′-5′ dibromo sulfanilanilide and succinyl sulfanilamide were not inhibitory in the amounts used; their bacteriostatic concentration is more than 8 mg %. Sulfamerazine and sulfadiazine

TABLE IV.
Effect of Disodium Sulfone on Various Organisms After 72 Hours Incubation.

Mg % conc. of sulfone	*S. aureus*	*Str. agalactiae*	*C. pyogenes*	*S. typhimurium*	*E. coli*	*Br. abortus*	*Ps. aeruginosa*
2270	—	—	—	±	±	—	±
1135	0	0	0	0	0	0	3+
227	±	±	±	2+	±	+	0
114	2+	0	+	3+	+	2+	0
23	0	±	0	0	0	0	0
11	0	+	0	0	0	0	0
Control	3+	3+	3+	3+	3+	3+	3+

0—No determination run.
— No growth, culture killed.
± Slight growth with no visible change in milk.
+, 3+ Relative amount of growth.

The 2.27% solution of disodium sulfone was obtained through the courtesy of Dr. C. A. Woodhouse from Dupont of Delaware, Md.

TABLE V.
Effect of Sodium Sulfamethazine on *S. agalactiae* and *E. coli*.

Mg % conc. of drug	Hours incubation	*St. agalactiae* strains 1723	MP5	24D	N-6	*E. coli* MP S
2500	24	—	—	—	—	—
	48	+	+	+	+	+
250	24	3+	+	3+	2+	+
	48	3+	3+	3+	3+	3+
25	24	3+	3+	3+	2+	+
	48	3+	3+	3+	3+	3+
2.5	24	3+	3+	3+	2+	+
	48	3+	3+	3+	3+	3+
Control	24	3+	3+	3+	3+	3+

1723, MP5, 24D, and N-6; *S. agalactiae* cultures from cows with chronic mastitis.
MP8—*E. coli* culture from a cow with acute mastitis.
— No change in milk.
+ Slight acid in milk.
2+ Acid in milk.
3+ Acid and coagulation in milk.

were active against *E. coli, Br. abortus, Str. agalactiae,* and *Ps. aeruginosa*. They appeared to have a wider range of action than the other sulfonamides. Sulfapyridine and sulfamethazine were slightly less active. Sulfone was much less active against the gram negative organisms. The strains of *S. aureus* and *S. typhimurium* were resistant to the sulfonamides. In Table IV the effect of disodium sulfone is presented. The concentration required for inhibition of growth was more than 227 mg % for all 7 strains. Disodium sulfone was more active against *Str. agalactiae* than against the other organisms. As shown in Table V sulfamethazine sodium inhibited the 4 *Str. agalactiae* cultures and one *E. coli* culture for 24 hours in a 2500 mg % concentration but permitted growth in this concentration in 48 hours. *E. coli* was slightly more sensitive than strains of *Str. agalactiae*.

Discussion and summary. Data are presented on the effect of various antibacterial agents on a representative group of organisms associated with cases of chronic and acute mastitis.

Subtilin was effective in high dilution against the gram positive organisms. Streptomycin appeared to more active against the gram positive cultures than against the gram negative organisms. Strain variations among cultures of the same species of organism may have been responsible for the poor action of streptomycin against these particular gram negative organisms.

The strains of *S. aureus* and *S. typhimurium* were resistant to the sulfonamides employed. *Str. agalactiae* and *C. pyogenes* were susceptible to sulfapyridine, sulfone, sulfadiazine and sulfamerazine. *In vivo* study is necessary to prove these drugs of value as adjuncts to penicillin in the treatment of chronic streptococcic mastitis and so-called "summer mastitis." *E. coli* was sensitive to sulfapyridine, sulfamethazine, sulfadiazine, and sulfamerazine among which sulfamerazine and sulfamethazine showed great activity. *Br. abortus* was susceptible to sulfamethazine, sulfadiazine, and sulfamerazine. *Ps. aeruginosa* was inhibited by sulfamerazine and sulfapyridine. Against the strain of *Ps. aeruginosa* employed, sulfadiazine was the most effective antibacterial agent.

16866

The Effect of Heparin on Cell Division.*

L. V. HEILBRUNN AND W. L. WILSON.

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When a living cell is incited to divide, its protoplasm first undergoes a sharp increase in viscosity. There is indeed what may be called a mitotic gelation and this gelation precedes the appearance of the mitotic spindle. Once the spindle is formed, the protoplasm reverts to its original fluid state. This sequence of stages in the protoplasm of a dividing cell has been described by all authors who have used objective methods for the determination of protoplasmic viscosity, (for discussion, see Heilbrunn;[1] Heilbrunn and Wilson[2]).

Since 1928,[1] it has been maintained that the mitotic gelation is similar to the gelation produced in protoplasm by so-called stimulating agents, and that this fundamental gelation reaction of protoplasm is in many respects similar to the gelation that occurs in vertebrate blood when it clots. As a matter of fact the protoplasm of all types of living cells is rich in thrombin or thromboplastic substances. substances which affect not only protoplasm but blood as well (compare Heilbrunn *et al.*[3] and references cited there).

If this line of reasoning is correct, it might be thought that substances which inhibit blood clotting would also prevent protoplasmic clotting in general and mitotic gelation in particular. Clearly, then, a substance like heparin is of interest in this connection.

Perhaps the best type of cell in which to observe protoplasmic clotting is the egg of the sea-urchin Arbacia. When this cell is torn or broken, as the protoplasm begins to flow out, the cell seals itself in a reaction like that which occurs when blood flows from a vessel.[4] This reaction has been called the surface precipitation reaction. Years ago, one of us attempted to show that heparin might have some effect on this surface precipitation reaction. However, no effect could be observed. Because of this failure to demonstrate an effect of heparin on the surface precipitation reaction, no further studies were made. Moreover, it was hardly to be expected

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* Aided by a grant from the U. S. Public Health Service.

[1] Heilbrunn, L. V., *The Colloid Chemistry of Protoplasm*. Berlin, 1928.

[2] Heilbrunn, L. V., and Wilson, W. L., *Biol. Bull.*, 1948, 95, 57.

[3] Heilbrunn, L. V., Harris, D. L., Le Fevre, P. G., Wilson, W. L., and Woodward, A. A., *Physiol. Zool.*, 1946, 19, 404.

[4] Heilbrunn, L. V., *Arch. f. exp. Zellforsch.*, 1927, 4, 246.

TABLE IV.
Effect of Disodium Sulfone on Various Organisms After 72 Hours Incubation.

Mg % conc. of sulfone	*S. aureus*	*Str. agalactiae*	*C. pyogenes*	*S. typhimurium*	*E. coli*	*Br. abortus*	*Ps. aeruginosa*
2270	—	—	—	±	±	—	±
1135	0	0	0	0	0	0	3+
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Control	3+	3+	3+	3+	3+	3+	3+

0—No determination run.
— No growth, culture killed.
± Slight growth with no visible change in milk.
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The 2.27% solution of disodium sulfone was obtained through the courtesy of Dr. C. A. Woodhouse from Dupont of Delaware, Md.

TABLE V.
Effect of Sodium Sulfamethazine on *S. agalactiae* and *E. coli*.

Mg % conc. of drug	Hours incubation	*St. agalactiae* strains				*E. coli* MP8
		1723	MP5	24D	N-6	
2500	24	—	—	—	—	—
	48	+	+	+	+	+
250	24	3+	+	3+	2+	+
	48	3+	3+	3+	3+	3+
25	24	3+	3+	3+	2+	+
	48	3+	3+	3+	3+	3+
2.5	24	3+	3+	3+	2+	+
	48	3+	3+	3+	3+	3+
Control	24	3+	3+	3+	3+	3+

1723, MP5, 24D, and N-6; *S. agalactiae* cultures from cows with chronic mastitis.
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were active against *E. coli*, *Br. abortus*, *Str. agalactiae*, and *Ps. aeruginosa*. They appeared to have a wider range of action than the other sulfonamides. Sulfapyridine and sulfamethazine were slightly less active. Sulfone was much less active against the gram negative organisms. The strains of *S. aureus* and *S. typhimurium* were resistant to the sulfonamides. In Table IV the effect of disodium sulfone is presented. The concentration required for inhibition of growth was more than 227 mg % for all 7 strains. Disodium sulfone was more active against *Str. agalactiae* than against the other organisms. As shown in Table V sulfamethazine sodium inhibited the 4 *Str. agalactiae* cultures and one *E. coli* culture for 24 hours in a 2500 mg % concentration but permitted growth in this concentration in 48 hours. *E. coli* was slightly more sensitive than strains of *Str. agalactiae*.

Discussion and summary. Data are presented on the effect of various antibacterial agents on a representative group of organisms associated with cases of chronic and acute mastitis.

Subtilin was effective in high dilution against the gram positive organisms. Streptomycin appeared to more active against the gram positive cultures than against the gram negative organisms. Strain variations among cultures of the same species of organism may

polar bodies can indicate whether or not sperm entrance has occurred. Because the polar bodies of Chaetopterus are rather small, it is not easy to make polar body counts. We made counts in two ways. If the eggs are undisturbed during the count, ordinarily only about a third of the polar bodies are visible, so that a count which showed approximately 35% of the eggs with polar bodies would indicate that all of the eggs had been fertilized. If the eggs are rolled during counting, as high as 90% of the eggs could be seen to have polar bodies. Ordinarily, we did not roll the eggs, and in calculating percentages of eggs with polar bodies, we assumed that a 35% count indicated that 100% of the eggs had polar bodies.

By making counts of polar bodies we were able to determine with some certainty whether or not sperm entrance had occurred in any given case. After some disappointing experiments, we were fortunate to find that by increasing the concentration of sperm we could override the inhibiting effect of heparin on fertilization. In some cases in which sperm entrance did not occur (as judged by the polar body test) we were able to induce sperm entrance by adding more sperm. As an example of the effect of sperm concentration, the following experiment may be cited. A drop of dry sperm, obtained by cutting a worm, was diluted with 10 ml of sea-water to form a sperm suspension. Varying amounts of this sperm suspension (1, 10, 20 drops) were added to eggs which had been for 10 minutes in a 0.05% solution of heparin in sea-water. The results are shown in Table I. In this table percentages of eggs with polar bodies are calculated as noted above. It is clear that higher concentrations of sperm cause an increased percentage of sperm penetration.

If sperm penetration has occurred and then the eggs do not cleave, it is obvious that heparin has prevented the division of the cell. Table II shows a series of 10 experiments. In all of these experiments, control eggs inseminated in sea-water showed approximately 100% fertilization and cleavage. The experimental eggs were inseminated after 10 minutes immersion in 0.05% heparin solutions. Polar body counts showed the approximate percentage of eggs in which sperm penetration had occurred, calculated as before. Of these fertilized eggs, only a relatively small percentage showed cleavage. From the table it is obvious that dilute solutions of heparin can block mitosis in the Chaetopterus egg.

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No. of drops of sperm suspension added	% of eggs with polar bodies
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10	51
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100	27	27
86	8	10
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51	25	49
57	10	18
51	36	71
63	6	10
100	13	13
91	42	46
100	61	61

In Table II the first 8 experiments were done with Hynson, Westcott and Dunning heparin (lot 198). In the final 2 experiments, Hoffmann-LaRoche heparin was used. Other experiments in which eggs were exposed to heparin for longer times indicated that Hoffmann-LaRoche heparin is weaker in its effects than Hynson, Westcott and Dunning heparin. Fortunately, however, the Hoffmann-LaRoche heparin does not appear to contain any thromboplastic substance.

The effect that heparin has on cell division is not due to a killing action; at any rate the effect is, to some extent at least, reversible. Thus in one experiment in which eggs treated with heparin for 10 minutes before fertilization showed 36% cleavage, washing the eggs several times in sea-water caused the percentage of cleavage to increase to 55%.

Apparently, heparin acts by preventing the mitotic gelation which normally occurs in the early stages of mitosis. In the Chaetopterus that heparin could act on the interior of a cell, for the heparin molecule is a large one and it scarcely seemed plausible to suppose that heparin could enter a cell in sufficient amount to affect clotting processes in the interior protoplasm.

Fortunately for us, some months ago, one of our beginning graduate students, Mr. Julius H. Jacobson, was less certain of failure and in some preliminary experiments was able to show that heparin tended to prevent clotting in the protoplasm of paramecium. Following this lead we took courage and began to wonder whether heparin might not have an effect on the mitotic gelation as it occurs in marine eggs.

In a recent paper[2] we have shown the exact course of the mitotic gelation in the egg of the worm Chaetopterus and the time sequence of this gelation in relation to the morphological changes which take place during cell division. Hence it was logical to attempt a study of the effect of heparin on various aspects of mitosis in the Chaetopterus egg.

Unfortunately, no very pure preparations of heparin were available to us. We began work with two different lots of the Hynson, Westcott and Dunning preparation. In preliminary experiments with sea-urchin (Arbacia) eggs, we found that one of these lots (no. 194) was apparently contaminated with thromboplastin or thromboplastic substance. When sea-urchin eggs are placed in oxalate solutions and then broken by pressing down on the coverslip over them, no surface precipitation reaction occurs and instead of the protoplasm sealing itself as it does when calcium is present, it flows out in a steady stream. On the other hand, if to the oxalate solution an extract of injured living material is added, the protoplasm even in the absence of calcium does show a surface precipitation reaction.[3] Thus the protoplasm of the sea-urchin egg can be used as an indicator for the presence of clotting (thromboplastic) substances. A drop of Arbacia eggs in sea-water was placed on a slide; to this was added a drop of 0.45 molar potassium oxalate and a drop of 0.25% solution of heparin from lot 194 of Hynson, Westcott and Dunning. After waiting a minute for the calcium of the sea-water to precipitate out, the eggs were crushed. A definite surface precipitation reaction occurred. The experiment was repeated many times, always with the same result. The result indicates the presence of a protoplasmic clotting substance in the heparin. An alcoholic extract of the heparin was prepared. This was evaporated to dryness and the dry residue dissolved in distilled water. The protoplasmic clotting substance was present in the alcoholic extract. Years ago, in preparing heparin from lung tissue, Charles, Fisher and Scott[5] found that it was difficult to separate the heparin from a substance in lung tissue which favored blood clotting; this clotting substance was soluble in alcohol. The presence of a clotting substance in lot 194 of the Hynson, Westcott and Dunning heparin was also indicated by the fact that dilute solutions (0.1 - 0.02%) also caused a well-marked increase of the protoplasmic viscosity of the protoplasm of sea-urchin eggs exposed to these solutions for 2 hours. Viscosity tests were made with the centrifuge method.

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When Chaetopterus eggs are placed in dilute heparin solutions and are inseminated some minutes later in the usual way with dilute suspensions of spermatozoa, they typically do not cleave. Much of this effect is due to an inhibition of fertilization by the heparin. Unfortunately, fertilization in the Chaetopterus egg is not accompanied by any marked cortical change, so that it is not possible to tell immediately whether or not sperm entrance has been effected. However, at about 9 minutes after fertilization (at 21°) the first polar body is given off, so that a count of

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Apparently, heparin acts by preventing the mitotic gelation which normally occurs in the early stages of mitosis. In the *Chaetopterus* egg, during the mitosis which causes the division of the single egg cell into two blastomeres, the protoplasmic viscosity rises sharply just before the mitotic spindle is formed. This is clearly shown in the graph published recently.[2] Before the mitotic gelation, the protoplasmic viscosity has an arbitrary value of 7; this rises to 14 and then after the spindle is formed it drops to 7 again. In the heparinized eggs, such a mitotic gelation does not occur. Five tests of the protoplasmic viscosity of fertilized heparinized eggs showed in every case a viscosity of 6 or below. These tests were all made from 70-90 minutes after fertilization, that is to say, after ample time had been allowed for the mitotic gelation to occur. Thus beyond much doubt heparin prevents mitosis by inhibiting the mitotic gelation.

The fact that heparin can prevent cell division is not a new observation. Years ago Fischer[6] showed that heparin could prevent the division of cells in tissue culture. Presumably in these cells also heparin acts by preventing protoplasmic clotting. The concentration of heparin that Fischer used in his later experiments is identical with that we found best for Chaetopterus eggs. However, Fischer apparently used a purer preparation of heparin than the one we had at our disposal.

An important fact to remember is that heparin is a normal constituent of the blood and perhaps also of the cells of higher animals. It has been stated that in radiation sickness there is an increased amount of heparin in the blood.[7] Conceivably some of the effect of roentgen rays on cancer might be due to the presence of this heparin and its effect on cell division. Also it should be noted that the bacterial polysaccharide which in minute amounts causes regression of tumors in rats and mice is a hemorrhagic agent.[8] Inasmuch as excessive doses of heparin are known to induce hemorrhage,[9] the bacterial polysaccharide may perhaps be related to heparin. It may also be possible that the injection of one type of heparin or another, or the injection of other types of substances which impede blood clotting, like injection of dicumarin, may eventually prove to be of some help in the prevention of excessive cell division such as occurs in cancer.

Recent experiments in our laboratory by Drusilla Harding have shown that heparin can prevent cell division in the frog egg. These experiments will be published before long. We are also experimenting on the effect of injecting heparin and other anti-coagulants on sarcoma in mice.

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[6] Fischer, A., *Arch. f. path. Anat. u. Physiol.*, 1930, **270**, 94; *Protoplasma*, 1936, **26**, 344.

[7] Allen, J. G., and Jacobson, L. O., *Science*, 1947, **105**, 388.

[8] Shear, M. J., and Turner, F. C., *J. Nat. Cancer Inst.*, 1943, 4, 81; Shear, M. J., Perrault, A., and Adams, J. R., *ibid.*, 1943, 4, 99; Hartwell, J. L., Shear, M. J., and Adams, J. R., *ibid.*, 1943, 4, 107.

[9] Erschler, I. L., and Blaisdell, I. H., *J. Amer. Med. Assn.*, 1941, **117**, 927; Richmond, E. L., *ibid.*, 1941, **118**, 609; Keyes, J. W., and Shaffer, C. F., *ibid.*, 1942, **110**, 882; Sollman, T., *A Manual of Pharmacology*, 3rd ed., Philadelphia, 1948, p. 425.

16867

Heparin and Heparinocytes in Elephantiasis Scroti.

WILLIAM E. EHRICH, JOSEPH SEIFTER, HARVEY E. ALBURN, AND ALBERT J. BEGANY.
From the Philadelphia General Hospital, the Graduate School of Medicine of the University of Pennsylvania, and the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

Through the brilliant researches of Jorpes[1] and Holmgren and Wilander[2] it has been well established that the mast cells or heparinocytes are cellular sources of heparin. However, heparin of the basophilic granules is not identical with circulating heparin or heparin extracted from tissues. The mast cell heparin is monosulfuric acid, and when released is transformed into di, tri, and tetrasulfuric acids, circulating or tissue heparin being a mixture of all of these elements.[3,4]

The distribution of heparinocytes in the tissues varies considerably in different species. In dogs, abundant heparinocytes are found in the lobules and veins of the liver, whereas in rabbits and rats they are scanty or absent here. In rats, abundant heparin is found in the skin. The intestines of the various animals all contain many heparinocytes; the lungs show a moderate number, and the spleen only a few. Rats contain fewer but larger heparinocytes than rabbits and dogs.[2,5]

It has been shown that heparin and similar compounds if given intravenously are taken up by the macrophages, particularly of the liver, spleen and lymph nodes, and that some is excreted through the kidneys.[6] That this is not the cause for the dissipation of heparin is apparent from studies involving splenectomy; complete hepatectomy; combined nephrectomy, splenectomy, and hepatectomy; injections into the bone marrow of the tibia; ligation of the carotid and vertebral arteries; blockade of the reticulo-endothelial system with India ink; stimulation of the reticulo-endothelial system by histamine, and depression of it by antihistaminic drugs.[7,8]

The pathology of the heparinocytes has hardly been studied. It is known only that the tissue mast cells are greatly increased in urticaria pigmentosa in man[9] and in solitary "mastocytomas" in man[9] and in dogs.[10] Oliver, Bloom and Mangieri,[11] who recently analyzed two dog mastocytomas chemically, found 20.1 g of a crude heparin in 330 g of tissue from a well differentiated tumor; from this they extracted 2,460,000 A.C.U. or 492,000 I.U. of heparin per kilogram of tumor, *i.e.*, 50 times as much as found in the liver of normal dogs. Paff, Bloom and Reilly[12] succeeded in cultivating these mast cells; they made the interesting observation that only mast cells grew in the cultures, as though the product elaborated by these cells prevented growth of other cells.

The present report deals with mast cells and heparin in a human scrotum amputated because of elephantiasis. The study was prompted by the observation of absence of thrombosis in this disease. It was speculated that coagulation of lymph and blood in the obstructed tissue was possibly prevented by excess heparin produced locally.

The owner of the scrotum, a white pastry

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[1] Jorpes, J. E., Heparin, Oxford University Press, 1946.

[2] Holmgren, H., and Wilander, O., *Z. mikr. anat. Forsch.*, 1937, **42**, 242.

[3] Jorpes, J. E., and Gardell, S., *J. Biol. Chem.*, 1948, **176**, 267.

[4] Jorpes, J. E., Werner, B., and Aberg, B., *J. Biol. Chem.*, 1948, **176**, 277.

[5] Wilander, O., *Skand. Arch. Phys.*, 1938-9, **81**, Suppl. 15.

[6] Asplund, J., Borell, U., and Holmgren, H., *Z. mikr. anat. Forsch.*, 1939, **46**, 15.

[7] Begany, A. J., and Seifter, Joseph, *Fed. Proc.*, 1948, **7**, 206.

[8] Seifter, J., and Begany, A. J., *Am. J. Med. Sci.*, 1948, **216**, 334.

[9] Sutton, R. L., and Sutton, R. L., Jr., Diseases of the Skin, St. Louis, 1939.

[10] Bloom, F., *Arch. Path.*, 1942, **33**, 661.

[11] Oliver, J., Bloom, F., and Mangieri, C., *J. Exp. Med.*, 1947, **88**, 107.

[12] Paff, G. H., Bloom, F. H., and Reilly, C., *J. Exp. Med.*, 1947, **86**, 117.

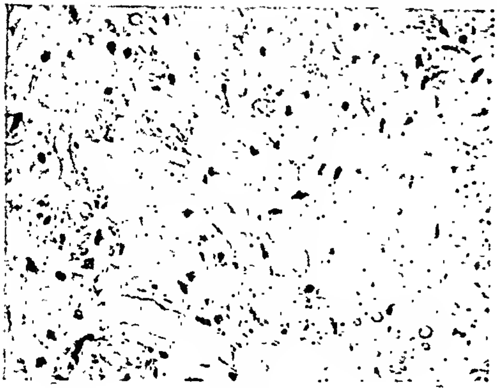

FIG. 1.
Abundant mast cells (heparinocytes) between distended lymph spaces.

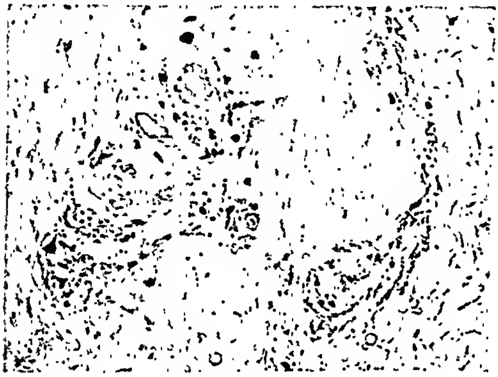

FIG. 2.
Abundant mast cells (heparinocytes) around blood and lymph vessels.

chef 55 years of age, came from Italy when he was 10. At 24, draft board examiners noted firm masses in both inguinal regions. When he was 43, the patient observed enlargement of the scrotum. He visited a physician only when the scrotum weighed over 50 lbs. and almost touched the floor. There was an invagination where the penis would normally be. The skin of the legs was congested and slightly cyanotic. There were superficial varicosities of all leg and thigh veins. The bleeding time was one minute, and the clotting time 5½ minutes.

Microscopic examination of tissue from the scrotum revealed numerous heparinocytes in the neighborhood of lymph and blood vessels (Fig. 1 and 2).

From 10 kg of tumor tissue, 11.11 g of crude heparin were extracted, following described methods.[13,14] Preparation of both the barium and the sodium salt yielded four fractions totalling 1.4 g of purified material. Plasma clotting studies were performed on thawed citrate dog plasma, using the photoelectric method as described by Foster.[15] One fraction of the heparin preparation in a concentration of 30 gamma per ml inhibited the clotting of plasma for 10 minutes, as opposed to 2½ minutes for the normal. Thirty gamma of a second fraction per ml prolonged the clotting of rabbit whole blood for 385 minutes, as compared with a control clotting time of 4 minutes. Intravenous injection of 5 mg of tumor heparin per kg into rabbits produced marked anticoagulation which reached its maximum within 15 minutes. A blood sample taken 15 minutes after the injection clotted in 65 minutes. A sample taken an hour after injection clotted in the normal time of 3 minutes.

The most potent fraction both *in vitro* and by direct intravenous injection into rabbits was of the same potency as the best commercial heparin currently used in clinical practice. The remaining fractions showed activity to a lesser degree. The fact that these fractions were not capable of purification to higher potency probably resides in the fact that most of the material was obtained from the mast cells and corresponded to Jorpes' lower sulfur-containing sulfuric acids, as the mono- and the di-sulfuric acid.

Summary and conclusions. Microscopic examination of a human scrotum amputated because of elephantiasis showed numerous heparinocytes around lymph and blood vessels. One hundred and twenty-six mg of purified heparin per kg of wet tissue was obtained. Based on the purified material, the scrotum contained at least 16,380 I.U. of activity per kg of fresh tissue. These findings are further evidence that mast cells are sources of heparin. They also explain the absence of thrombosis in elephantiasis.

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[13] Charles, A. F., and Scott, D. A., *Tr. Roy. Soc. Canada*, sect. 5, 1934, **28**, 55.

[14] Kuizenga, M. H., and Spaulding, L. B., *J. Biol. Chem.*, 1943, **148**, 641.

[15] Foster, R. H. K., *Am. J. Physiol.*, 1948, **152**, 577.

16868

Survival Differences Breathing Air and Oxygen at Equivalent Altitudes.[*][†]

HERMANN RAHN AND ARTHUR B. OTIS.

From the Department of Physiology and Vital Economics, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

It can be shown on theoretical grounds[1] that when equivalent altitudes are chosen where the pO_2 of the inspired gases are identical breathing air and breathing 100% oxygen the resultant *alveolar* pO_2 in the lung are never the same unless the respiratory quotient is equal to unity. For example, if the alveolar pCO_2 is 40 mm Hg and the R.Q. = .8, the alveolar pO_2 is 8 mm lower breathing air than breathing 100% oxygen. Under the same conditions but with the R.Q. = 1.0 the alveolar pO_2 is the same. When the R.Q. is greater than 1.0 the alv. pO_2 is larger breathing air than breathing 100% oxygen. Thus at equivalent inspired O_2 tension altitudes the relative advantage or disadvantage of breathing air as compared to 100% oxygen is a function of the R.Q. This phenomenon has been referred to as the *R.Q. Effect* or *Nitrogen Dilution Effect* (the latter applies more specifically to conditions of R.Q. greater than unity). Acute exposure to low oxygen concentration induces hyperventilation which raises temporarily the respiratory quotient above 1.0. As long as the quotient remains above 1.0 it would be preferable to breath air rather than 100% oxygen at equivalent inspired O_2 pressures. Theoretically an advantage of only a few mm in alveolar pO_2 pressure is expected, but since in very low hypoxic environments a few mm pO_2 spell the difference between consciousness and unconsciousness it was felt that this nitrogen dilution effect might be demonstrable.

Method. Mice were conditioned to sit on a horizontal bar of ½" diameter suspended about 8 cm above the base of a large desiccator. The bottom was provided with a metal grid which electrically shocked the animal whenever it left the bar. Mice are very readily conditioned to this procedure and will immediately jump on the bar even when no current is sent through the grid. *Unconsciousness* was designated as that state where mice were no longer able to maintain themselves on the bar. This criterion has proven to be a very satisfactory index. The oxygen pressure was reduced by evacuating the desiccator by a pump. The rates of ascent on air and 100% O_2 had to be very accurately controlled so that the rates of change of the inspired pO_2 (B.T.P.S.) were identical in the two runs. Thus the timing of air runs started at ground level where the inspired pO_2 was .209 x (B-47). In the 100% O_2 runs the desiccator was first thoroughly flushed with oxygen for several minutes. This was followed by a slow ascent to an altitude of approximately 32900 ft. to allow for denitrogenation. The timing of the pure oxygen runs was started at this altitude where the inspired pO_2 (equal to B-47) was the same as at ground level. Usually 6 mice were "taken up" at one time and the inspired pO_2 was calculated from the altitude at which unconsciousness set in. The air temperature on all runs was kept between 22.9 and 24.1°C.

Since the N_2 dilution or R.Q. effect is a transient affair depending upon the degree of hyperventilation and the magnitude of the CO_2 reserve of the blood and tissues, the rate of ascent was varied as indicated in Table I. The rates of ascent so listed are based upon 100% oxygen runs which started at 32900 ft. The ascents on air were adjusted in such a

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[1] Fenn, W. O., Rahn, H., and Otis, A. B., *Am. J Physiol.*, 1946, **146**, 637.

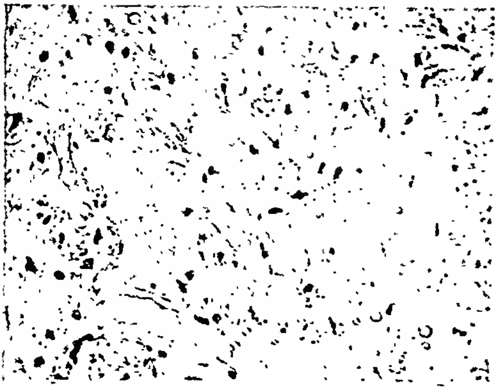

FIG. 1.
Abundant mast cells (heparinocytes) between distended lymph spaces.

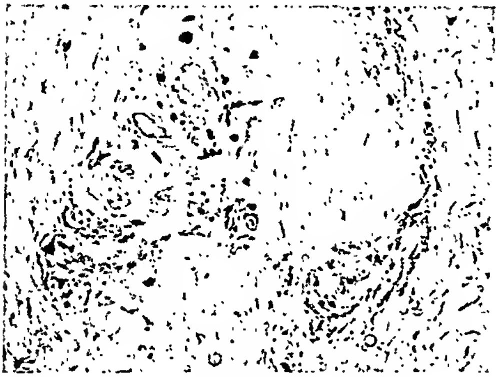

FIG. 2.
Abundant mast cells (heparinocytes) around blood and lymph vessels.

chef 55 years of age, came from Italy when he was 10. At 24, draft board examiners noted firm masses in both inguinal regions. When he was 43, the patient observed enlargement of the scrotum. He visited a physician only when the scrotum weighed over 50 lbs. and almost touched the floor. There was an invagination where the penis would normally be. The skin of the legs was congested and slightly cyanotic. There were superficial varicosities of all leg and thigh veins. The bleeding time was one minute, and the clotting time 5½ minutes.

Microscopic examination of tissue from the scrotum revealed numerous heparinocytes in the neighborhood of lymph and blood vessels (Fig. 1 and 2).

From 10 kg of tumor tissue, 11.11 g of crude heparin were extracted, following described methods.[13,14] Preparation of both the barium and the sodium salt yielded four fractions totalling 1.4 g of purified material. Plasma clotting studies were performed on thawed citrate dog plasma, using the photoelectric method as described by Foster.[15] One fraction of the heparin preparation in a concentration of 30 gamma per ml inhibited the clotting of plasma for 10 minutes, as opposed to 2½ minutes for the normal. Thirty gamma of a second fraction per ml prolonged the clotting of rabbit whole blood for 385 minutes, as compared with a control clotting time of 4 minutes. Intravenous injection of 5 mg of tumor heparin per kg into rabbits produced marked anticoagulation which reached its maximum within 15 minutes. A blood sample taken 15 minutes after the injection clotted in 65 minutes. A sample taken an hour after injection clotted in the normal time of 3 minutes.

The most potent fraction both *in vitro* and by direct intravenous injection into rabbits was of the same potency as the best commercial heparin currently used in clinical practice. The remaining fractions showed activity to a lesser degree. The fact that these fractions were not capable of purification to higher potency probably resides in the fact that most of the material was obtained from the mast cells and corresponded to Jorpes' lower sulfur-containing sulfuric acids, as the mono- and the di-sulfuric acid.

Summary and conclusions. Microscopic examination of a human scrotum amputated because of elephantiasis showed numerous heparinocytes around lymph and blood vessels. One hundred and twenty-six mg of purified heparin per kg of wet tissue was obtained. Based on the purified material, the scrotum contained at least 16,380 I.U. of activity per kg of fresh tissue. These findings are further evidence that mast cells are sources of heparin. They also explain the absence of thrombosis in elephantiasis.

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[13] Charles, A. F., and Scott, D. A., *Tr. Roy. Soc. Canada*, sect. 5, 1934, **28**, 55.

[14] Kuizenga, M. H., and Spaulding, L. B., *J. Biol. Chem.*, 1943, **148**, 641.

[15] Foster, R. H. K., *Am. J. Physiol.*, 1948, **152**, 577.

16868

Survival Differences Breathing Air and Oxygen at Equivalent Altitudes.[*][†]

HERMANN RAHN AND ARTHUR B. OTIS.

From the Department of Physiology and Vital Economics, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

It can be shown on theoretical grounds[1] that when equivalent altitudes are chosen where the pO_2 of the inspired gases are identical breathing air and breathing 100% oxygen the resultant *alveolar* pO_2 in the lung are never the same unless the respiratory quotient is equal to unity. For example, if the alveolar pCO_2 is 40 mm Hg and the R.Q. = .8, the alveolar pO_2 is 8 mm lower breathing air than breathing 100% oxygen. Under the same conditions but with the R.Q. = 1.0 the alveolar pO_2 is the same. When the R.Q. is greater than 1.0 the alv. pO_2 is larger breathing air than breathing 100% oxygen. Thus at equivalent inspired O_2 tension altitudes the relative advantage or disadvantage of breathing air as compared to 100% oxygen is a function of the R.Q. This phenomenon has been referred to as the *R.Q. Effect* or *Nitrogen Dilution Effect* (the latter applies more specifically to conditions of R.Q. greater than unity). Acute exposure to low oxygen concentration induces hyperventilation which raises temporarily the respiratory quotient above 1.0. As long as the quotient remains above 1.0 it would be preferable to breath air rather than 100% oxygen at equivalent inspired O_2 pressures. Theoretically an advantage of only a few mm in alveolar pO_2 pressure is expected, but since in very low hypoxic environments a few mm pO_2 spell the difference between consciousness and unconsciousness it was felt that this nitrogen dilution effect might be demonstrable.

Method. Mice were conditioned to sit on a horizontal bar of ½" diameter suspended about 8 cm above the base of a large desiccator. The bottom was provided with a metal grid which electrically shocked the animal whenever it left the bar. Mice are very readily conditioned to this procedure and will immediately jump on the bar even when no current is sent through the grid. *Unconsciousness* was designated as that state where mice were no longer able to maintain themselves on the bar. This criterion has proven to be a very satisfactory index. The oxygen pressure was reduced by evacuating the desiccator by a pump. The rates of ascent on air and 100% O_2 had to be very accurately controlled so that the rates of change of the inspired pO_2 (B.T.P.S.) were identical in the two runs. Thus the timing of air runs started at ground level where the inspired pO_2 was .209 x (B-47). In the 100% O_2 runs the desiccator was first thoroughly flushed with oxygen for several minutes. This was followed by a slow ascent to an altitude of approximately 32900 ft. to allow for denitrogenation. The timing of the pure oxygen runs was started at this altitude where the inspired pO_2 (equal to B-47) was the same as at ground level.

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I	0.5	23.3	12.2	23	24.6	1.7	23	—1.3	2.40
II	2.	41.7	7.6	33	32.5	5.6	35	9.2	1.63
III	4.	39.8	7.0	28	31.0	3.5	33	8.8	1.45
IV	16.	40.8	6.5	22	31.7	3.7	22	9.1	1.60

fashion that the inspired pO_2 at any time was equal to that of the O_2 runs.

Results and Discussion. Four different rates of ascent were tried. In Series I it took on the average 42 minutes to reach the unconsciousness level and there was no significant difference between the two runs. It might be argued that during this long interval the N_2 dilution effect was nearly over (the CO_2 reserves used up) as has been found for altitudes of 18-22000 in man.[2] Had a still slower rate of ascent been used where the R.Q. had time to return to its true metabolic value the tolerance on O_2 should have been better than on air. In such a case a N_2 concentration effect would have obtained.

In Series 2, 3 and 4 the rate of ascent was increased so that unconsciousness was reached after 6, 3 and 1 minutes respectively. It was felt that some of these ascent rates should catch the maximum N_2 dilution effect. This one would expect to be maximal at the beginning of hypoxic ventilation. It can be seen that all 3 rates gave approximately the same difference in pO_2 level at which unconsciousness was produced. All these differences are highly significant as indicated by the standard error of the differences between the means as shown in the last column of Table I. The pO_2 difference can also be expressed in terms of altitude. In other words, if the N_2 dilution effect were not present in the air runs then the unconscious level would have been reached at an altitude of 28500 ft. (inspired pO_2 of 41) instead of 33000 ft. (inspired pO_2 of 31).

It should be pointed out that another factor might enter into the discrepancy of these two runs. That is the possible occurrence of aeroembolism in the oxygen runs which by itself may contribute to the lower pO_2 threshold. Furthermore, the criterion of the inspired pO_2 at B.T.P.S. ($T = 37°C$) is probably not strictly true at very low hypoxic levels when the metabolism and the body temperature sink rapidly in these small animals. This temperature drop would increase the pO_2 tension. However, this should affect both series and if effective at all would favor the 100% O_2 animals.

Summary. Mice were exposed progressively to equivalent degrees of hypoxia breathing air and breathing 100% O_2. The inspired pO_2 level at which unconsciousness set in was found to be significantly lower when air was breathed than when 100% oxygen was breathed provided the rate of ascent was 2×10^3 ft/min or greater. This advantage of air over oxygen is attributed to the transient "N_2 dilution" or R.Q. effect.

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16869

Studies on the Localization of Tagged Methionine Within the Pancreas.[*]

James E. Wheeler, F. D. W. Lukens, and Paul György.

From the Department of Pediatrics, the Gastro-Intestinal Section of the Medical Clinic, and the George S. Cox Institute, School of Medicine, University of Pennsylvania.

Recent studies of intermediary metabolism with isotopic compounds[1,2] have amply confirmed the concept of Schoenheimer[3] that tissue proteins are in a dynamic state of equilibrium with amino acids of exogenous source. It has been shown that different tissues show different activities in this respect,[4] and that intestinal mucosa is particularly active. Friedberg[5] postulated that the secretion of digestive enzymes and muco-proteins, which are rapidly lost from the tissue, would account for this rapid metabolic rate. On this basis he predicted that pancreas would show high activity, a fact actually shown earlier by Tarver and Schmidt.[6]

In balance studies, Gordon[7] showed that when labelled methionine, prepared with isotopic, radioactive sulphur (S^{35}), was given orally to animals, a high percentage of radioactive sulphur was recovered from pancreas, small intestine and liver. It was thought that such concentrations represent rapid incorporation of methionine into digestive enzymes; however, a possible role in the synthesis of hormones, particularly of insulin, cannot be excluded by ordinary balance studies. Attempts to demonstrate more accurate localization of isotopic compounds within tissues by radioautography have thus far been unsatisfactory in our hands. The present experiments attempt to localize the site of early methionine metabolism in the pancreas.

Experimental. Radioactive sulphur (S^{35}) was incorporated in methionine[†] starting with soluble sulphate. The specific activity of the final product (crystalline dl-methionine) approximated one microcurie per milligram (on date of receipt), based on a radioassay by another laboratory. In all cases this was administered intravenously in 2 ml water, and Table I shows the dosages given.

Preparation of and findings in the 4 cats in Table I were as follows:

No. 1 A normal cat. *Autopsy*: Pancreas was grossly normal; no sections were made.

No. 2 The pancreatic duct was ligated at the duodenum. Postoperative course was uneventful, and the animal gradually gained weight. Methionine was given 104 days after duct ligation. *Autopsy*: Grossly, there was complete late atrophy of the duodenal portion of the pancreas, partial atrophy of the tail of the pancreas (it is believed than an accessory duct preserved this portion). No sections were made.

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TABLE I.
Doses of Labelled Methionine Given and Content* of Radioactive Sulphur Found in the Pancreas.

Cat No.	Wt, kg	Methionine given, microcuries, per kg	Normal pancreas		Atrophic pancreas
			4 hr	8 hr	8 hr
1	2.1	2.4	2970	3000	
2	2.9	1.7			1560†
3	3.1	2.4		2610	101
4	3.6	2.1		1500	8.1

* Standard specific activity (× 10^4).

† From portion of pancreas which showed incomplete atrophy.

ligature. Sections showed normal pancreas proximal to the ligature; in the ligated portion, as seen in Fig. 1, there was patchy and incomplete atrophy of acinar tissue with occasional islets. The preservation of portions of acinar tissue is attributed to incomplete ligation.

No. 4 A ligature was placed around the body of the pancreas in its midportion. Again, postoperative course was uneventful, and weight was regained by the time of administration of labelled methionine 125 days after ligation. *Autopsy:* The pancreas appeared grossly normal proximal to the ligature, but there was apparent complete atrophy distal to the ligature. Sections showed normal pancreas proximal to the ligature. In the ligated portion, as seen in Fig. 2, there was marked atrophy of acinar tissue with intact islets, surrounded by fibrous tissue and lymphocytes.

All animals showed normal blood sugar concentrations after these operative procedures. In all cases methionine was given under light nembutal anesthesia. In Cats No. 1 and No. 2 pancreatic and liver biopsies were performed by laparotomy 4 hours after administration of methionine. All animals were sacrificed 8 hours after administration of methionine, and the organs and tissues were weighed, frozen and subsequently assayed.

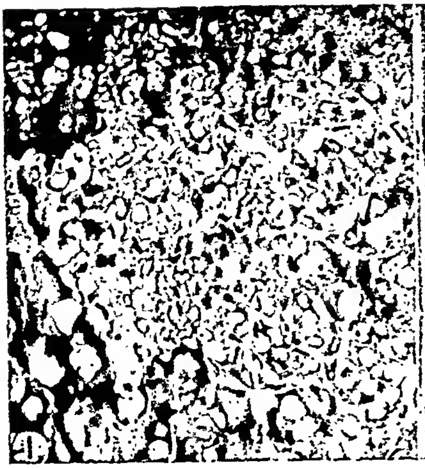
The tissues were oxidized by digestion in Kjeldahl flasks containing Pirie reagent (perchloric acid-nitric acid-copper nitrate mixture), and soluble sulphate was precipitated with benzidine dihydrochloride. Sulphur content was determined by alkali titration, and sulphate was subsequently precipitated as barium sulphate. This precipitate was filtered with standardized apparatus on weighed paper, and radioactivity was directly measured with a thin-window, bell-type Geiger counter, the sensitivity of which was established by assay of a sample of known radioactivity. All dosages and counts for this laboratory are based on this standard. Assayed counts were corrected to a standard weight of barium sulphate, and were corrected for decay, based on the original assay. All results are based on duplicate analyses.

The radioactivity of samples, expressed as Standard Specific Activity (S.S.A.),[4] was calculated as follows, and is shown in Table II:

$$\text{S.S.A.} = \frac{100 \times \text{assayed counts} \times \text{weight of animal (kg)}}{\text{mEq sulphur in sample} \times \text{counts in dose}}$$

Discussion. It has been known since the work of Laguesse that the islet cells of the duct-ligated portion of a pancreas are physiologically intact, a fact which is supported in these animals by the morphological appearance of the pancreas. It would appear that the low concentration of radioactive sulphur found in tissue from the ligated portion, as compared with that from normal pancreas (Table I), is due to loss of acinar tissue. On this basis it appeared that the rapid early turnover of methionine by the pancreas is due to metabolic activity of the exocrine portion of the gland rather than to the needs of the islets. Some labelled methionine may be converted into cystine, which may then be incorporated in insulin by islet tissue, but, if so, this represents a small percentage of the unexpectedly high concentration of activity found in normal pancreas. The high concentrations found in liver, duodenum and jejunum (Table II) confirm the results of other investigators.

Summary. Methionine labelled with radioactive sulphur (S^{35}) was given intravenously to 4 cats, one normal, 3 treated so as to pro-



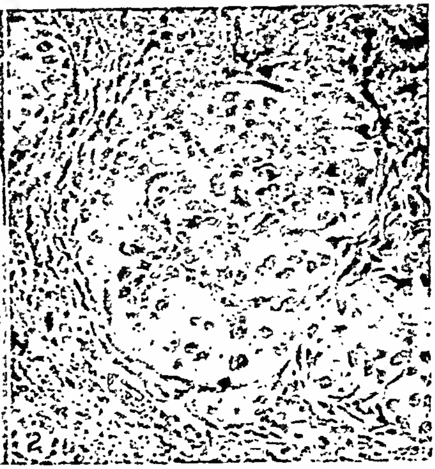


FIG. 1. Island of Cat 3. The cells of the island show some vacuolation and degranulation. Partial atrophy of the acinar tissue about the island is shown by the vacuolation and distortion of these cells.

FIG. 2. Island of Cat 4. The normal island is surrounded by fibrous tissue and lymphocytes. Atrophy of acinar tissue was complete in this portion of the animal's pancreas.

TABLE II.
Concentration of Radioactive Sulphur in Various Tissues.*

Cat No.	Duodenum	Jejunum	Liver	Bile	Kidney	Ovary	Testis
1	2185		1430		721		
2	1120		1540		684	77.1	
3	1440	882	1370		471	170	
4	840	672	848	528	699		16

* Expressed as standard specific activity ($\times 10^4$)

duce varying degrees of atrophy of the acinar tissue of the pancreas. The animals were sacrificed 8 hours after methionine was given, and various tissues were assayed for radioactive sulphur.

In all animals high activity was found in pancreas, small intestine and liver. In the atrophic portion of the pancreas where a ligature had been placed around the body of that gland, low activity was found as contrasted with high activity found in the normal part of the gland. Where atrophy was incomplete, the activity found was intermediate between that of normal and atrophic tissue.

It is concluded that the high activity of radioactive sulphur found in the pancreas of normal cats after administration of labelled methionine represents principally the metabolic activity of the exocrine portion of the pancreas.

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ligature. Sections showed normal pancreas proximal to the ligature; in the ligated portion, as seen in Fig. 1, there was patchy and incomplete atrophy of acinar tissue with occasional islets. The preservation of portions of acinar tissue is attributed to incomplete ligation.

No. 4 A ligature was placed around the body of the pancreas in its midportion. Again, postoperative course was uneventful, and weight was regained by the time of administration of labelled methionine 125 days after ligation. *Autopsy*: The pancreas appeared grossly normal proximal to the ligature, but there was apparent complete atrophy distal to the ligature. Sections showed normal pancreas proximal to the ligature. In the ligated portion, as seen in Fig. 2, there was marked atrophy of acinar tissue with intact islets, surrounded by fibrous tissue and lymphocytes.

All animals showed normal blood sugar concentrations after these operative procedures. In all cases methionine was given under light nembutal anesthesia. In Cats No. 1 and No. 2 pancreatic and liver biopsies were performed by laparotomy 4 hours after administration of methionine. All animals were sacrificed 8 hours after administration of methionine, and the organs and tissues were weighed, frozen and subsequently assayed.

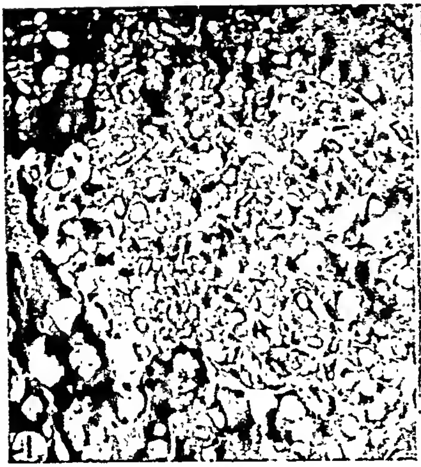
The tissues were oxidized by digestion in Kjeldahl flasks containing Pirie reagent (perchloric acid-nitric acid-copper nitrate mixture), and soluble sulphate was precipitated with benzidine dihydrochloride. Sulphur content was determined by alkali titration, and sulphate was subsequently precipitated as barium sulphate. This precipitate was filtered with standardized apparatus on weighed paper, and radioactivity was directly measured with a thin-window, bell-type Geiger counter, the sensitivity of which was established by assay of a sample of known radioactivity. All dosages and counts for this laboratory are based on this standard. Assayed counts were corrected to a standard weight of barium sulphate, and were corrected for decay, based on the original assay. All results are based on duplicate analyses.

The radioactivity of samples, expressed as Standard Specific Activity (S.S.A.),[4] was calculated as follows, and is shown in Table II:

$$\text{S.S.A.} = \frac{100 \times \text{assayed counts} \times \text{weight of animal (kg)}}{\text{mEq sulphur in sample} \times \text{counts in dose}}$$

Discussion. It has been known since the work of Laguesse that the islet cells of the duct-ligated portion of a pancreas are physiologically intact, a fact which is supported in these animals by the morphological appearance of the pancreas. It would appear that the low concentration of radioactive sulphur found in tissue from the ligated portion, as compared with that from normal pancreas (Table I), is due to loss of acinar tissue. On this basis it appeared that the rapid early turnover of methionine by the pancreas is due to metabolic activity of the exocrine portion of the gland rather than to the needs of the islets. Some labelled methionine may be converted into cystine, which may then be incorporated in insulin by islet tissue, but, if so, this represents a small percentage of the unexpectedly high concentration of activity found in normal pancreas. The high concentrations found in liver, duodenum and jejunum (Table II) confirm the results of other investigators.

Summary. Methionine labelled with radioactive sulphur (S^{35}) was given intravenously to 4 cats, one normal, 3 treated so as to pro-



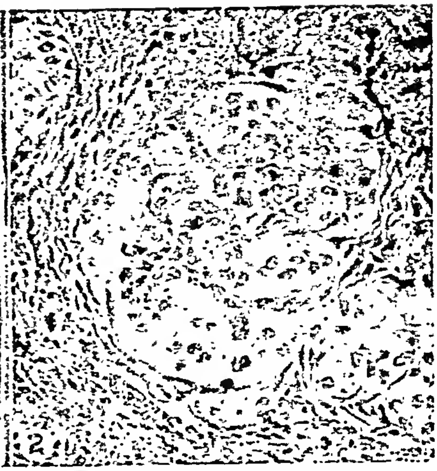


FIG. 1. Island of Cat 3. The cells of the island show some vacuolation and degranulation. Partial atrophy of the acinar tissue about the island is shown by the vacuolation and distortion of these cells.

FIG. 2. Island of Cat 4. The normal island is surrounded by fibrous tissue and lymphocytes. Atrophy of acinar tissue was complete in this portion of the animal's pancreas.

TABLE II.
Concentration of Radioactive Sulphur in Various Tissues.*

Cat No.	Duodenum	Jejunum	Liver	Bile	Kidney	Ovary	Testis
1	2185		1430		721		
2	1120		1540		684	77.1	
3	1440	882	1370		471	170	
4	840	672	848	528	699		16

* Expressed as standard specific activity ($\times 10^4$)

duce varying degrees of atrophy of the acinar tissue of the pancreas. The animals were sacrificed 8 hours after methionine was given, and various tissues were assayed for radioactive sulphur.

In all animals high activity was found in pancreas, small intestine and liver. In the atrophic portion of the pancreas where a ligature had been placed around the body of that gland, low activity was found as contrasted with high activity found in the normal part of the gland. Where atrophy was incomplete, the activity found was intermediate between that of normal and atrophic tissue.

It is concluded that the high activity of radioactive sulphur found in the pancreas of normal cats after administration of labelled methionine represents principally the metabolic activity of the exocrine portion of the pancreas.

(Continued from page iv)

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16870

Comparison of Synthetic and Fermentation Chloramphenicol (Chloromycetin) in Rickettsial and Viral Infections.

Joseph E. Smadel, Elizabeth B. Jackson, Herbert L. Ley, Jr., and Raymond Lewthwaite.

From the Army Medical Department Research and Graduate School and the Commission on Immunization of the Army Epidemiological Board, Washington, D.C., and the Institute for Medical Research, Kuala Lumpur.

Synthetic chloromycetin, recently prepared by Crooks and his coworkers,[1] has been shown to have the same physical properties and antibacterial activities as chloromycetin produced by the mold *Streptomyces venezuelae*, N. Sp. The present report provides comparative data on the rickettsiostatic and virustatic effects of crystalline chloromycetin obtained from two sources, *i.e.*, from fermentation liquor of cultures of *Streptomyces venezuelae*, N. Sp.[2] and from chemical synthesis.[1] Both types were supplied by the Research Laboratories of Parke, Davis and Company. Earlier publications have provided information on the use of the fermentation type of drug in the treatment of rickettsial and viral infections of experimental animals[3-7] and man.[8-11]

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[1] Controulis, J., Rebstock, M. C., and Crooks, H. M., Jr., *J.A.C.S.*, 1949, in press.

[2] Bartz, Q. R., *J. Biol. Chem.*, 1948, **172**, 445.

[3] Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

[4] Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, **106**, 417.

[5] Smith, R. M., Joslyn, D. A., Gruhzit, O. M., McLean, I. W., Jr., Penner, M. A., and Ehrlich, J., *J. Bact.*, 1948, **55**, 425.

[6] Smadel, J. E., Jackson, E. B., and Cruise, A. B., *J. Immunol.*, in press.

[7] Smadel, J. E., and Jackson, E. B., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 478.

[8] Smadel, J. E., León, A. P., Ley, H. L., Jr., and Varela, G., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 12.

TABLE I.
Comparison of Effect of Two Types of Chloromycetin in Infected Embryonated Eggs.

Infecting agent	Drug dose mg/egg	Mean prolongation of life in days of embryos treated with chloromycetin	
		Fermentation	Synthetic
R. rickettsi	.125	1.65	1.78
	.25	2.91	2.91
R. akari	.125	1.89	2.06
	.25	3.67	3.48
R. mooseri	.125	1.23	1.09
	.25	2.26	2.63
R. prowazecki	.125	1.20	1.00
	.25	2.82	2.82
R. tsutsugamushi (Gilliam strain)	.125	1.02	1.07
	.25	3.20	3.09
(Seerangayee strain)	.25	2.30	2.40
Psittacosis virus (6 BC strain)	.25	1.63	1.83
	.50	3.33	3.63
(P 4 strain)	.25	1.29	1.45
	.50	3.20	2.96
Lymphogranuloma virus (J.H. strain)	.25	3.93	3.89
	.50	7.12	7.23

Methods. The methods employed in the present work were similar to those used in previous studies[3,6-8,10] and need not be discussed.

Results. Table I summarizes the data obtained in embryonated eggs treated with varying doses of the 2 types of chloromycetin one-half hour before inoculation with a given rickettsial or viral strain. In this work the causal agents of epidemic, murine and scrub typhus, spotted fever and rickettsialpox, and of psittacosis and lymphogranuloma venereum were employed. Inspection of Table I reveals that the mean prolongations of life (MPL) of the groups infected with a given agent and treated with the same amounts of fermentation or synthetic drug were essentially identical. Statistical analysis of the basic data confirmed this. In these tests the MPL represented the difference between the mean day of death of infected embryos in treated and control groups. Each group contained 20-24 living embryos at the beginning of the period for calculating the mean day of death; depending on the infectious agent, deaths within the first 3 or 4 days after inoculation were considered non-specific.

Mice infected with the Karp strain of *R. tsutsugamushi* received a single daily intraperitoneal injection of solutions containing varying amounts of the 2 types of drug. The results obtained in one such test in which treatment was begun the day following injection of rickettsiae and continued for 20 days are summarized in Table II. Both types of drug were highly effective in preventing death of the animals.

In other experiments mice were infected intraperitoneally with the 6 BC strain of psittacosis virus and treated by intraperitoneal injection or oral administration of varying doses of the 2 types of chloromycetin. The results were essentially indistinguishable from those previously obtained[7] when the fermentation

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[9] Payne, E. H., Knaudt, J. A., and Palacios, S., *J. Trop. Med. and Hyg.*, 1948, **51**, 68.

[10] Smadel, J. E., Woodward, T. E., Ley, H. L., Jr., Philip, C. B., Traub, R., Lewthwaite, R., and Savoor, S. R., *Science*, 1948, **108**, 160.

[11] Pincoffs, M. C., Guy, E. G., Lister, L. M., Woodward, T. E., and Smadel, J. E., *Ann. Int. Med.*, 1948, **29**, 656.

TABLE II.
Comparison of Chemotherapeutic Effect of Two Types of Chloromycetin in Mice Infected with *R. tsutsugamushi*.

Drug treatment I. P.		Dilution of infectious inoculum		
Type	mg/day/mouse	10^{-5}	10^{-6}	10^{-7}
None	(Controls)	8/8*	4/8	4/8
Fermentation	2.5	0/8		
	1.5	0/8		
	0.75	0/8		
	0.375	2/8		
Synthetic	2.5	0/8		
	1.5	0/8		
	0.75	1/8		
	0.375	3/8		

* Numerator = No. of mice dying; denominator = No. of mice in group.

All mice in control groups receiving 10^{-2}, 10^{-3}, 10^{-4} dilutions of challenge material succumbed while one died in the 10^{-8} group. The titer of the inoculum was $10^{-6.6}$, therefore, the drug treated mice received approximately 40 MLD's intraperitoneally.

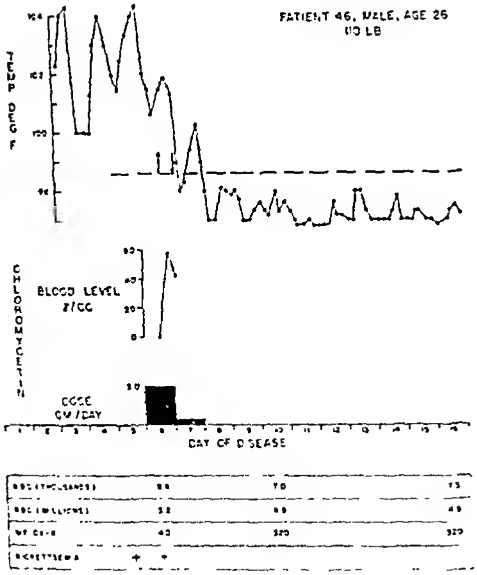

FIG. 1.

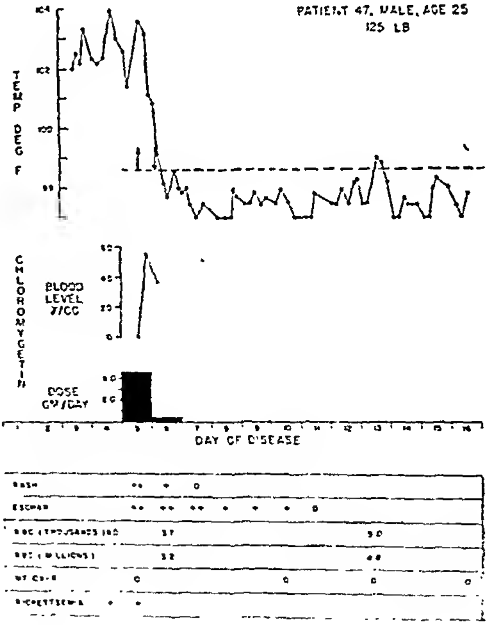

FIG. 2.

type of antibiotic was employed under similar conditions. Chloromycetin from both sources was equally virustatic in the present tests.

The clinical findings in 2 patients with scrub typhus* who were treated with synthetic chloromycetin are summarized in Fig. 1 and 2. The diagnosis was established in each individual by recovering *R. tsutsugamushi* on 2 occasions from samples of blood which were drawn during the febrile phase and promptly injected intraperitoneally into mice. Furthermore, during convalescence Patient 46 developed agglutinins for the OX-K strain of *B. proteus*. Both patients were considered to have had typical scrub typhus although neither presented all the classical signs of the disease. Thus, Patient 46 failed to show a rash or eschar while Patient 47 failed to develop a positive Weil-Felix reaction. Both persons received the drug orally over a period of 16 hours. Patient 46 was given an initial dose of 3.0 g followed by 4 doses of 0.25 g at 3-hour intervals. The regime for Patient 47 was similar except that the initial amount was 4.0 g. The response of these 2 patients following therapy was entirely comparable to that observed in other cases of scrub typhus treated with fermentation chloromycetin. It will be recalled that the febrile period of scrub typhus, which usually lasts for 14 days in untreated cases, was terminated in 31 hours (average for 25 patients) after treatment with chloromycetin was instituted.[10]

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* The authors wish to thank Major C. J. Williams, RAMC, and other members of the Staff of the Military Hospital, Kuala Lumpur, who were responsible for the care of these patients.

Our experience, although limited, indicates that synthetic chloromycetin possesses the same low level of toxicity for embryonated eggs, mice and men that has characterized the fermentation type[3,5,10] of drug.

Conclusion. Chloromycetin prepared by chemical synthesis appears to possess the same rickettsiostatic and virustatic properties in experimental infections and the same usefulness in treating patients with scrub typhus that have been demonstrated for chloromycetin produced by *Streptomyces venezuelae*, N. Sp.

16871

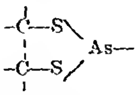
Condensation of 2, 3-Dimercaptopropanol (BAL) with Oxophenarsine Hydrochloride: Toxicity and Chemotherapeutic Effect.

John L. Sawyers, Benjamin Burrows, and Thomas H. Maren.*
(Introduced by E. K. Marshall, Jr.)

From the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University.

The use of 2,3-dimercaptopropanol (BAL) in the treatment of arsenical poisoning has been well established, and the pharmacological and biochemical basis for its action has been thoroughly explored. It has been shown that BAL mobilizes arsenic from tissues in which the metal has undergone a reversible binding with sulfhydryl groups in protein.[1,2] As a result BAL increases the excretion of administered arsenic from the body[3,4] and is a useful antidote in poisoning from arsenical gases or complications following administration of oxophenarsine hydrochloride.[2-7]

It has been shown that when arsenic reacts with keratin the ratio of combination with sulfur of the protein is 1 As : 2 S, and this suggested that a relatively stable ring of the type



is formed.[1] It is therefore postulated that BAL detoxifies by the removal of arsenic from the protein system and incorporation *in vivo* into a more stable cyclic

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[*] Eli Lilly Fellow in Pharmacology and Experimental Therapeutics.

[1] Stocken, L. A., and Thompson, R. H. S., *Biochem. J.*, 1946, **40**, 529.

[2] *ibid.*, p. 535.

[3] *ibid.*, p. 548.

[4] Eagle, H., Magnuson, H. J., and Fleischman, R., *J. Clin. Invest.*, 1946, **25**, 451.

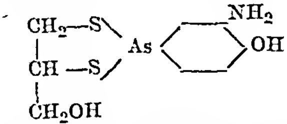
[5] Longcope, W. T., Luetscher, J. A., Jr., Wintrobe, M. M., and Jagen, V., *J. Clin. Invest.*, 1946, **25**, 528.

[6] Carleton, A. B., Peters, R. A., Stocken, L. A., Thompson, R. H. S., and Williams, D. I., *J. Clin. Invest.*, 1946, **25**, 497.

[7] Durlacker, S. H., Bunting, H., Harrison, H. E., Ordway, N. K., and Albrink, W. S., *J. Pharmacol. and Exp. Therap.*, 1946, **87**, Supplement, 28.

thioarsenite with BAL itself.[2,8]

Three papers[9-11] have appeared dealing with some of the pharmacological properties of the thioarsenite which is formed *in vitro* by condensation of oxophenarsine hydrochloride and BAL. For convenience, the compound will be called BAL-OXO. Its structure is as follows:



However, there is a conflict concerning the toxicity of this compound relative to the parent oxophenarsine hydrochloride. Peters and Stocken,[9] using a propylene glycol solution of the hydrochloride of BAL-OXO, found that in rats it was approximately four times as toxic as the parent arsenoxide. Excess BAL reduced this toxicity markedly. On the other hand, Riker[11] found in cats that BAL-OXO was at least 4 times less toxic than the parent compound. Friedheim and Vogel[10] gave toxicity figures of BAL-OXO for the mouse and the rabbit which are considerably lower than those published elsewhere for oxophenarsine hydrochloride.[12] No direct comparison was made however. These workers also found that BAL-OXO was therapeutically active in mouse trypanosomiasis and rabbit syphilis. Although no quantitative evaluation of the drug was made, they suggested that it might be a useful chemotherapeutic agent.

The present experiments were designed to explore the relative toxicity, and therapeutic value in *T. equiperdum* in mice, of BAL-OXO and the parent oxophenarsine hydrochloride.

Materials and methods. A single strain of mice weighing 20-25 g was used throughout. Rats used for toxicity weighed 120-160 g. Animals were from Carworth Farms, Inc.

Drugs were administered in a single dose intraperitoneally. In the therapeutic experiments they were given 24 hours after a suspension of approximately 100,000 organisms of *T. equiperdum* was injected into the peritoneal cavity of the mice.

The oxophenarsine hydrochloride was a commercial preparation containing 31.8% arsenic.[†]

BAL-OXO was prepared as follows: To 1.88 g (.008 mols) of oxophenarsine hydrochloride in 100 ml N HCl was added 1 g (.008 mols) of 2,3-dimercaptopropanol in 20 ml methanol. Sodium hydroxide (3N) was added until neutrality was reached. A copious light pink precipitate was formed and filtered under suction. The precipitate was washed with water, methanol and ether, and dried in air. The compound was a fine light pink powder, with a melting point of 122-124°C. Analysis showed an arsenic content of 24.5% and 24.6%. The theoretical value is 24.6%. This compound was used within 3 months after it was made. There was no darkening or observable change in solubility characteristics. For solution and use in animals the following procedure was followed: 100 mg drug was dissolved in 12 ml water and 3 ml N HCl, with gentle heating. Normal NaOH was then added until the solution was only slightly acid (approx. 2.5 ml used), but no precipitate had formed. It was made up to appropriate volume and used in this form, which is the hydrochloride of BAL-OXO.

Doses of both compounds are expressed in terms of mg of arsenic per kg body weight.

In toxicity experiments all animals were observed for 14 days. Almost all deaths occurred in the first 48 hours, after oxophenarsine hydrochloride. Deaths from BAL-OXO were often delayed for 96 hours. Mice in therapeutic tests were observed for 30 days. Most deaths were in the first two weeks and none occurred beyond the twentieth day. Ten untreated infected controls died within 5 days.

Results were plotted as per cent survival against the logarithm of the dose for both toxicity and therapeutic experiments. In Fig.

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[8] Whittaker, V. P., *Biochem. J.*, 1947, 41, 56.

[9] Peters, R., and Stocken, L. A., *Biochem. J.*, 1947, 41, 53.

[10] Friedheim, E. A. H., and Vogel, H. J., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 418.

[11] Riker, W. F., *J. Pharmacol. and Exp. Therap.*, 1946, 87, Supplement, 66.

[12] Eagle, H., Hogan, R., Doak, G. O., and Steinman, H. G., *Public Health Rep.*, 1944, 59, 765.

[†] We wish to thank Dr. A. C. Bratton of Parke, Davis Company for supplying this compound in a chemically pure state.

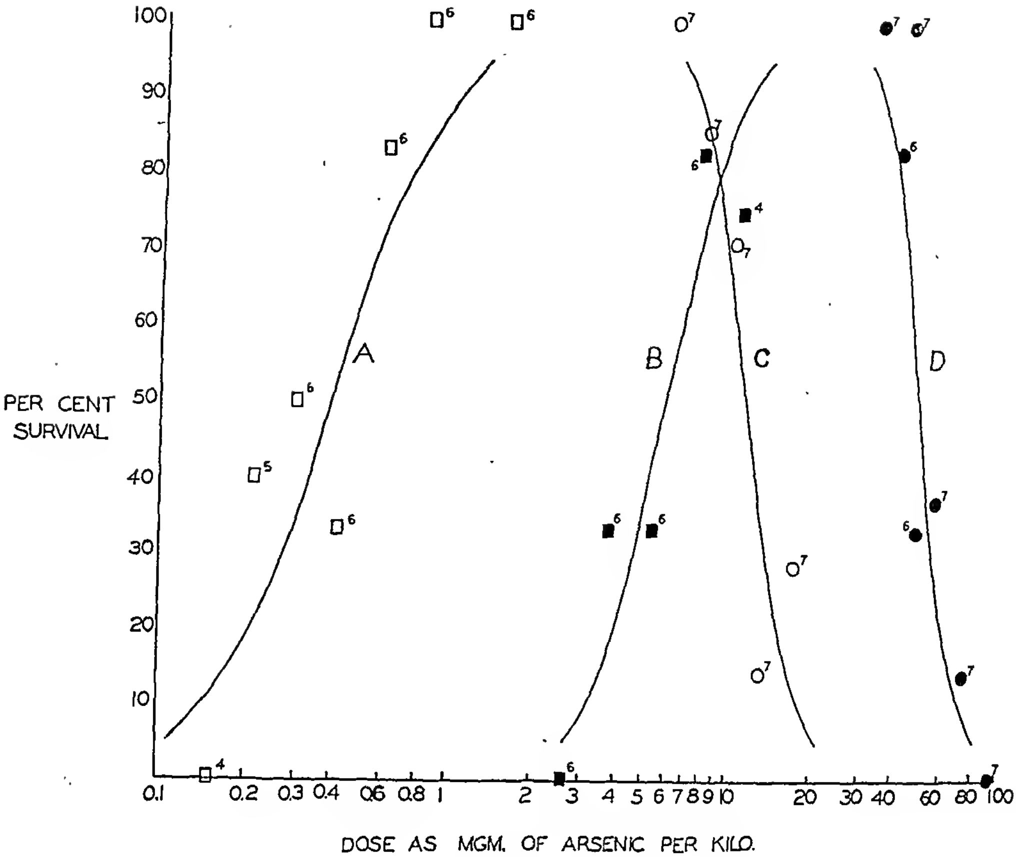

FIG. 1.

The mouse toxicity, and therapeutic effect of oxophenarsine HCl and BAL-oxo in *T. equiperdum* in mice.

Key:
□ Oxophenarsine HCl therapy, curve A.
■ BAL-oxo therapy, curve B.
○ Oxophenarsine HCl toxicity, curve C.
● BAL-oxo toxicity, curve D.

The small numbers on the figure represent the number of animals used at each dose.

1, integrated normal frequency curves have been fitted to the observed data. "Chemotherapeutic index" is defined as the dose lethal to 50% of animals/dose curing 50% = LD$_{50}$/CD$_{50}$.

Results. Fig. 1 shows the data obtained for 4 experiments on mice. Smooth curves to approximate these points were constructed as described above. The acute toxicity (curves C and D) experiments were run simultaneously for the two drugs. Approximately one month later the treatment of *T. equiperdum* was run simultaneously for both drugs (curves A and B). The approximate 50% survival point may be read from Fig. 1, and will give the LD$_{50}$ (curves C and D) and the CD$_{50}$ (curves A and B) for both compounds. Because of the comparatively small number of animals used no attempt was made to attach a standard error to these measurements. For oxophenarsine hydrochloride the ratio LD$_{50}$/CD$_{50}$ is approximately 30. This is in agreement with Eagle, Hogan, Doak and Steinman whose figure is 26.6.[12] The corresponding ratio for BAL-OXO is approximately 10.

It is evident therefore that the condensation of BAL with oxophenarsine hydrochloride yields a compound of lower "chemotherapeutic index" than the parent arsenoxide

The toxicity curves (C and D) show that

conversion of the arsenoxide to the thioarsenite has the effect of *decreasing* the acute toxicity in mice by a factor of 4. This agrees with Riker's data on cats.[11] Peters and Stocken,[9] who reported that this conversion *increased* the toxicity by approximately 4, worked entirely with rats. It was essential, therefore, to know if the disagreement was due to a species difference, and we set up several acute toxicity experiments on rats. For oxophenarsine hydrochloride, we were in fair agreement with Peters and Stocken who reported the LD_{50} as 5 mg As/kg. Our animals survived 6.5 mg As/kg and died at 10 mg As/kg. For BAL-OXO, however, there was an enormous disparity; our rats survived 32 mg As/kg and died at 64 mg As/kg, whereas Peters and Stocken reported that the LD_{50} was between 1-2 mg As/kg. It will be observed that our figures for rats agree well with our mouse data (curves C and D). Species difference alone, therefore, would clearly not account for the results of Peters and Stocken, which disagree with those of other workers, including ourselves.

Discussion. These findings suggest that in the combination of oxophenarsine hydrochloride with BAL the net toxicity for the host and for the parasite are not affected to the same degree. This agrees with Ercoli and Wilson[12] who found that relatively more BAL was needed to interfere with the toxicity of oxophenarsine hydrochloride than was needed to interfere with its trypanocidal and therapeutic effect. Both our findings and theirs, therefore, show that BAL lowers the therapeutic index of the arsenical.

The precise reason for this effect is not completely evident, but at least two hypotheses would explain the facts. First, the dithioarsenite may act as a whole and have a different relative toxicity for host and parasite than does oxophenarsine hydrochloride. On the other hand, BAL may simply alter the distribution of active arsenical between trypanosome and host. We believe that the latter view is more in keeping with all that is known of the action of arsenicals. If the dithioarsenite acts as a whole, it must act by a mechanism not explainable by the -SH-arsenoreceptor theory which is now generally accepted. For this reason, we believe that the oxophenarsine-BAL compound acts through dissociation into a compound whose arsenic is available to sulfhydryl groups in protein.

In any case, it has been confirmed that BAL-OXO does possess significant therapeutic activity, although its therapeutic index is only about 1/3 that of oxophenarsine hydrochloride. It is entirely possible that other arsenic-sulfhydryl compounds might show a greater therapeutic efficacy than the one we have studied, but from this single example it appears that combination of arsenical with a detoxifying dithiol reduces its therapeutic effect to a greater degree than its toxicity.

Regarding toxicity alone, the major discrepancy between our results and those of Riker[11] and Friedheim[10] on the one hand, and those of Peters and Stocken[9] on the other, are quite inexplicable. It is possible that differences in the preparation or stability of the drug are responsible. Secondly, they gave their compound in propylene glycol. A circumstance which suggests that Peters and Stocken were not dealing solely with the effect of the BAL-OXO is that on this drug their animals died faster than with oxophenarsine hydrochloride alone, and usually succumbed within 2 hours. In our experiments quite the reverse was noticed: even on relatively high doses, death following the thioarsenite was delayed over that of the arsenoxide.

Summary. 1. The condensation product of BAL and oxophenarsine hydrochloride (BAL-OXO) was studied. Its toxicity in mice and chemotherapeutic activity in *T. equiperdum* is reported, in comparison with similar data, for oxophenarsine hydrochloride.

2. The acute toxicity of the thioarsenite, BAL-OXO is approximately ¼ that of the arsenoxide (oxophenarsine hydrochloride) from which it was prepared.

3. The BAL-OXO has less therapeutic advantage, *i.e.,* lower LD_{50}/CD_{50}, in mouse trypanosomiasis than oxophenarsine hydrochloride itself.

16872

Requirement of the German Cockroach for Choline and Related Compounds.*

JERRE L. NOLAND AND C. A. BAUMANN.

From the Department of Biochemistry, School of Agriculture, University of Wisconsin, Madison,

Ordinarily choline is not considered a critical vitamin in insect nutrition, although its omission may result in retarded growth.[1] Recently, however, it was shown[2] that the omission of choline from the diet of newly hatched German roaches resulted in a complete cessation of growth within 10 days and the death of all of the nymphs within 30 days. The omission of each of the other vitamins singly resulted in less marked effects upon growth and survival.

In the present study the requirement of the roach *Blattella germanica* (L.) for choline was determined quantitatively with synthetic diets otherwise optimal for growth and maturation. Compounds related to choline were also studied. The 40-odd nymphs from a single egg sac were divided into groups of 5-7 and kept in wire-screened test-tube cages[3] at a temperature of 27-32°C. Each group received a different diet, based on variations of synthetic diet V (Table I). Food and water were given *ad libitum* and were replaced weekly, and the roaches were weighed under CO_2 anaesthesia at 10, 20, and 30 days of age. The age of maturation of each roach was also recorded.

Choline Requirement. Young roaches fed diet V (Table I) from which the choline was omitted failed to gain weight after the 10th day (Table II) and they all died before the 40th day. At a level of 500 γ of choline/g of diet V, the growth rate was very slow and only one-fifth of the insects reached maturity. At a level of 1000 γ of choline/g of diet the growth rate was improved but was still subnormal, although most of the insects matured. At both the levels of 2000 and 4000 γ choline /g of diet, growth was good, and the insects matured at an average age of 39 days, with practically all surviving. A further increase in the level of dietary choline to 8000 γ/g of diet resulted in suboptimal growth (Table II).

TABLE I.
Composition of Synthetic Diet V.

	Parts
Glucose monohydrate (*Cerelose*)	31
Casein ("vitamin-free")	30
*Cellu Flour**	30
Wesson's salt mixture†	4
Corn oil	3
Cholesterol	1
Vitamin mixture	0.6
	γ/g
Choline chloride	4000
Thiamine hydrochloride	12
Riboflavin	18
Nicotinic acid	100
Calcium pantothenate	40
Pyridoxine	16
Inositol	2000
Biotin	0.6
Folic acid	5

* Manufactured by Chicago Dietetic Supply House, Chicago, Ill.

† Wesson, L. G., *Science*, 1932, **75**, 339.

The finding that the cockroach requires as much as 2000 γ of choline/g of diet for optimal growth is in contrast to the experiments of Fraenkel and Blewett with other insect species[1] which were uniformly fed only 500 γ of choline/g diet. However, 2000 γ/g have been reported[4] necessary for good growth of chicks fed homocystine and a minimal level of methionine.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] Fraenkel, G., and Blewett, M., *Biochem. J.*, 1943, **37**, 686.

[2] Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc. Am.*, in press, II.

[3] Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc. Am.*, in press, I.

[4] McKittrick, D. S., *Arch. Biochem.*, 1948, **18**, 437.

TABLE II.
Growth, Maturation, and Survival of the Cockroach *Blattella germanica* on Synthetic Diets Containing Various Amounts of Choline.

No. of times fed	Choline Cl γ/g diet	Wt of roaches			Avg. age at matur. days	Survival at matur.
		10 days, mg	20 days, mg	30 days, mg		
2	0	4	3	4	dead	0/11
4	500	4	5	10	80	4/22
5	1000	6	16	33	44	26/28
7	2000	6	21	44	39	34/36
6	4000	6	21	44	39	35/35
1	8000	5	19	35	45	6/6

An effort was made to demonstrate alterations in choline requirement due to changes in the level of dietary fat, cholesterol, cystine, or niacinamide. Corn oil was included at levels of 1, 3 and 10% of the diet, and cholesterol was fed at levels of 0.3, 1 and 3% of the diet. Various combinations of these 3 levels of fat and cholesterol were fed in diets containing 500, 1000 and 4000 γ of choline/g diet with no significant differences in growth rate resulting. L-Cystine and niacinamide fed at the levels of 1% and 870 γ/g, respectively, in a diet containing 1000 γ choline/g diet, likewise failed to produce significant changes in growth or maturation. It was, therefore, concluded that the requirement of the roach for 2000-4000 γ of choline/g of diet is a reasonably constant one.

Activity of Other Compounds. DL-methionine, dimethylaminoethanol, aminoethanol, or betaine hydrochloride were added to diet V in the absence of choline or as supplements to diets containing a low level of the vitamin, 500 γ/g of diet. On the choline-free diet, methionine, dimethylaminoethanol and aminoethanol had very little growth-stimulating

TABLE III.
Response of the German Cockroach to Choline and Related Compounds Fed at Equimolar Levels in Synthetic Diet V.

Exp.*	γ/g diet		Wt of roaches			Avg. age at matur. days	Survival at matur.
			10 days, mg	20 days, mg	30 days, mg		
	Compounds added to choline-free diet.						
C, G	Choline Cl	1000	6	12	36	44	9/11
C, D	DL-methionine†	3210	3	6	5	dead	0/10
C, D	Dimethylamino-ethanol†‡	956	4	6	7	dead	0/10
C, D	Aminoethanol	438	4	6	7	dead	0/10
C, G	Betaine HCl	1100	6	13	36	45	10/12
D	Choline Cl	2000	5	19	46	40	5/5
D	Betaine HCl	2200	5	20	48	38	11/12
G	Choline Cl	4000	6	19	38	41	7/7
G, G	Betaine HCl	4400	6	18	33	43	13/14
	Compounds added to diet containing 500 γ of choline/g.						
A, B	None		4	6	12	78	2/10
A, B	Choline Cl	1500	6	20	40	42	8/10
A, B	DL-methionine	1610	5	10	20	72	5/10
A, B	Dimethylamino-ethanol	956	6	15	24	54	8/10
A, B	Aminoethanol	657	4	6	10	92	2/10
A, B	Betaine HCl	1650	6	22	41	40	10/10

* Dietary groups included in the same series were lettered the same.

† Added at levels equivalent to choline Cl on a methyl basis. In all other experiments the compounds were added at levels equivalent to choline Cl on a molar basis.

‡ Carbide and Carbon Chem. Corp. We are indebted to R. Grunert for this preparation.

TABLE IV.
Effect of Diet Upon the Choline Content of German Cockroaches, as Determined by the *Neurospora* Method.

Insects used		Diet		Choline content	
Sex	Age, days	Basal	Composition	Range γ/g live wt.	Avg
127 nymphs	3	crude	dog biscuits	900-1320	1080
2 M, 3 F	58	synth. V	choline Cl, 1000 γ/g	1520-1830	1620
3 M, 2 F	50	″ III*	″ Cl, 2000 γ/g	1630-1830	1730
2 M, 2 F	58	″ V	″ Cl, 4000 γ/g	2690-2880	2780
3 M, 3 F	58	″ V	betaine HCl, 1100 γ/g	1140-1480	1290
2 M, 3 F	58	″ V	″ HCl, 4400 γ/g	1890-2090	2020

* Same basal diet as synth. V except vitamin K and p-aminobenzoic acid were added.

effect, since the nymphs gained only 2 or 3 mg from the 10th to the 30th days of age (Table III), and all such nymphs died before maturing. Nymphs receiving 1000 γ of choline/g diet gained 30 mg during this time. When the diets contained betaine, however, growth was equal to that of nymphs receiving an equimolar amount of choline, and the growth-promoting activity of betaine was found to equal that of choline at all the levels tested.

When the various compounds were added to a diet containing 500 γ of choline/g at levels equivalent to 1500 γ/g of choline on a molar basis, aminoethanol remained completely inactive while both methionine and dimethylaminoethanol appeared to increase growth, maturation, and survival to a considerable extent although neither compound was as active as choline itself (Table III). On the other hand the roaches fed betaine grew at least as well and matured at the same rate as those receiving choline.

Choline Content of Roaches. The successful replacement of choline by betaine in the diet of the roach in spite of the high choline requirement of this species suggested a possible interconversion of these substances *in vivo*. Accordingly, newly-hatched nymphs, and roaches which had matured on diets containing varying levels of choline or betaine were analyzed for choline with the cholineless mutant of *Neurospora crassa* 34486. The insects were starved for 48 hours, anaesthetized with chloroform and chopped with scissors into a test tube containing 10 ml of 3% H_2SO_4, and the sample was treated as described by Horowitz and Beadle,[5] except that no attempt was made to remove methionine, since the amounts of this amino acid in the roaches were too small to interfere with the determination. The mold pads were filtered from the medium, washed with distilled water, rolled into pellets and dried on a porcelain plate for 6 hours at 90°C. before weighing. The standard curve covered the range 0-20 γ choline per flask. Essentially quantitative recoveries of choline were obtained in experiments in which choline and betaine were added separately or together to aliquots of freshly chopped insects, in agreement with the finding of Horowitz and Beadle that betaine is inactive for cholineless Neurospora.[5]

The newly-hatched nymphs were found to contain an average of 1080 γ choline/g live weight (Table IV) while adults maturing on diets containing 1000, 2000 and 4000 γ choline/g diet contained an average of 1620, 1730 and 2780 γ choline/g live weight respectively. Adults which had been fed diets containing 1100 and 4400 γ of betaine hydrochloride/g of diet instead of choline contained 1290 and 2020 γ of choline /g live weight respectively. Since the newly-hatched nymphs weighed an average of 2.26 mg each, they contained only 2.4 γ choline/insect. On the other hand the insects fed no choline but 1100 and 4400 γ of betaine/g diet weighed, on the average, 68.2 and 89.0 mg, and contained 88 and 180 γ choline/insect respectively. It is therefore evident that choline had been synthesized either by these insects or by the microorganisms associated with them. Roaches fed betaine at a molar equivalent of 1000 γ choline/g diet contained about 80% as much choline as insects fed choline itself; at a level of betaine equivalent to 4000 γ choline/g diet, they contained 73% as much body choline as those fed choline itself. The roaches fed the higher level of betaine contained 2020 γ of choline/g live weight whereas those fed 2000 γ of choline/g of diet, the optimal amount for growth and maturation, contained only 1730 γ of choline /g of live weight (Table IV). Thus, amounts of choline above those actually "needed" had accumulated in the betaine-fed group.

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[5] Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, 1943, **150**, 325.

Discussion. The relative inactivity of aminoethanol and dimethylaminoethanol in the absence of choline suggests that in the cockroach the methylation of aminoethanol and dimethylaminoethanol by dietary methionine[†] must be an inefficient process if, indeed, it occurs at all. This is in contrast to the finding that aminoethanol can be methylated by methionine in the rat,[6] and that dimethylaminoethanol plus methionine supports fair growth in the chick[7] in the absence of choline.

On the diet containing a minimal level of choline the feeding of methionine, dimethylaminoethanol, aminoethanol and betaine to roaches resulted in graded growth responses which were proportional to the methyl content of the supplements, with aminoethanol being completely inactive, methionine poor, dimethylaminoethanol fair, and betaine equal to choline. Apparently, therefore some of the functions of choline can be met by methionine and dimethylaminoethanol. In this respect, the cockroach may be similar to the chick in which choline has both an "essential" and a "replaceable" role.[4] The roach is different from the chick, however, in that betaine can completely replace choline in the diet of the roach whereas it meets only the "replaceable" needs for choline by the chick.[4]

In view of the efficient synthesis of body choline by cockroaches fed betaine, and the apparent difficulty of dietary aminoethanol and dimethylaminoethanol to be methylated by methionine, it is suggested that the mechanism of choline synthesis in the cockroach may be different from that proposed[8] for the rat, *viz.*, betaine→ glycine→ ethanolamine→ choline. Rather, a direct conversion of betaine to choline is suggested.

Summary. 1. The omission of choline from an otherwise adequate synthetic diet resulted in complete failure of roaches to grow and in death shortly thereafter. For optimal growth and maturation 2000-4000 γ of choline/g of diet were found to be needed.

2. The addition of dimethylaminoethanol or aminoethanol to diets lacking choline but containing 30% of casein resulted in little or no growth. The addition of methionine, dimethylaminoethanol and betaine as supplements to a diet low in choline resulted in an increased growth response in the order named. Aminoethanol was inactive.

3. Dietary betaine replaced choline quantitatively for growth and maturation at all levels of intake, and insects fed betaine contained nearly as much choline as those fed a corresponding amount of choline.

[†] Synthetic diet V contains 0.9% methionine as a constituent of casein (Table 1).

[6] duVigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, 1940, **134**, 787.

[7] Jukes, T. H., and Oleson, J. J., *J. Biol. Chem.*, 1945, **157**, 419.

[8] Stetten, DeWitt, Jr., *J. Biol. Chem.*, 1941, **140**, 143.

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2 M, 2 F	58	” V	” Cl, 4000 γ/g	2690-2880	2780
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TABLE II.
Microscopic Pathology in Livers of Animals on Diet I at Both Biopsies and at Autopsy.

Control group				Diet I		
Rat No.	*Fat*	*Fibrosis*		*Rat No.*	*Fat*	*Fibrosis*
1	0	0	Biopsy at 85-90 days	6	4+	0
2	0	0		7	4+	1+
3	0	0		8	4+	0
4	0	0		9	4+	0
5	0	0		10	3+	0
			Biopsy at 155-165 days	6	4+	1+
				7	4+	2+
				8	4+	1+
				9	4+	1+
				10	3+	0
1*	0	0	Autopsy at 190-200 days and animals dying earlier	6	4+	2+
2*	0	0		7	4+	2+
3*	0	0		8	4+	2+
4	2+	0		9	4+	2+
5*	0	0		10	4+	0

* Animals dying before 190 days.

Grading of Fat

1+ Few droplets in every lobule.
2+ <half of every lobule infiltrated.
3+ >half of every lobule infiltrated.
4+ Almost all of every lobule infiltrated.

Grading of Fibrosis

1+ Strands of fibrous tissue scattered throughout section.
2+ Tissue divided into pseudolobules by fibrous bands.

received a daily oral supply of thiamin, 20 γ; riboflavin, 25 γ; pyridoxine, 20 γ; and calcium pantothenate, 100 γ. Vitamins A and D were supplied by adding 5 drops of Oleum Percomorphum§ per kilogram of diet to the melted lard as the diet was prepared.

Diet 1. The rats weighed from 140 to 170 g and one group of 13 animals were fed Diet I, and a second group of 10 rats received the control diet (Table I). Water and food was allowed *ad libitum*, and daily food intake was measured in 5 rats of each group who were housed in individual cages. At 85-90 days liver biopsies were performed on all of the animals in the individual cages by laparotomy under ether anesthesia. The biopsy was taken from the edge of the left lobe of the liver. This procedure was repeated on the same animals in the same manner at 155-165 days. After 190-200 days on the diets the animals were sacrificed and specimens of tissue were taken from the center of the left and right lobes of the liver and from the kidney. The remaining liver of all animals was analyzed for total lipids by a modification of the method of Outhouse and Forbes.[13] All of the tissue sections were stained with hematoxylin and eosin and in addition sections stained with Sudan III were prepared from half of the animals in each group. In a few cases Masson, reticulin, acid fast stain and Best-carmine stain were also utilized.

Diet 2. Animals fed this diet weighed from 130 to 160 g. Ten rats received diet 2 and ten animals were fed the control diet (Table I). Food was allowed *ad libitum* and daily measurement of food intake was made on five rats of each group. No biopsies were taken on these animals. Six rats in each group were sacrificed after 150 days and the remainder on the 200th day of the experiment. Tissue sections were made as outlined under Diet I. Lipid analyses were made on half of the animals in each group.

Diet 3. The rats used weighed between 110 and 150 g. Ten animals were fed diet 3 and 10 received the control diet. All of the animals were housed in individual cages and each animal received *only* 8 g of food per day. The exact amount of food consumed by each animal was measured daily. Five

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§ The authors wish to thank Dr. C. E. Bills of Mead, Johnson and Company for the Oleum Percomorphum.

[13] Outhouse, E. L., and Forbes J. C., *J. Lab. and Clin. Med.*, 1939, **25**, 1157.

16873

Relation of Fat and Protein Intake to Fatty Changes, Fibrosis and Necrosis of the Liver.*

CHARLES A. HALL† AND VICTOR A. DRILL.‡

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It is still undetermined whether the lesions seen in the livers of animals on a low protein diet or a high fat diet are all part of the same process, or are the results of two or more distinct processes. Several investigators have produced hepatic lesions of fatty change, necrosis, and fibrosis by dietary means.[1-6] Others observed the fatty change and fibrosis but no necrosis.[7-9] Daft and coworkers[4] suggested that these hepatic changes which had been considered as all having the same pathogenesis should be divided into two processes, one resulting in a cirrhosis and the other a necrosis. Himsworth and Glynn[10] reported that diets high in fat or low in lipotropic substances resulted in a fatty change followed by a diffuse fibrosis of the liver, while diets low in protein but containing lipotropic substances resulted in a massive hepatic necrosis with post necrosis scarring in animals that survived. The necrosis could be prevented by adding methionine to the diet.[11] It was also reported that necrosis could be produced by feeding diets in which the protein was supplied by an amino acid mixture omitting methionine and cystine.[12] Again methionine and also cystine prevented the necrosis. This has led to the development of the theory that dietary liver disease in rats can be divided into one type consisting of fatty change and fibrosis which is produced by a diet high in fat or carbohydrate and deficient in lipotropic substances, and a second type of damage consisting of necrosis and scarring which results from a deficiency of sulfur containing amino acids. The present study was designed to further observe the effect of a high-fat diet, and a low-protein diet, on the type of liver injury produced.

TABLE I.
Experimental Diets.

	Control diet, %	Diet I, %	Diet II, %	Diet III, %
Casein	16	16	6	4
Lard	6	51	6	6
Cornstarch	60	30	70	72
Sucrose	15	—	15	15
Salt mixture	3	3	3	3
	100	100	100	100

Methods. The animals used were male, Sprague-Dawley rats. The composition of the diets fed is listed in Table I. Each rat also

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† Post-war Fellow in Pharmacology, 1947-48, Rockefeller Foundation.

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[1] Blumberg, H., and McCollum, E. V., *Science*, 1941, **93**, 598.

[2] Webster, G. T., *J. Clin. Invest.*, 1942, **21**, 385.

[3] György, P., and Goldblatt, H., *J. Exp. Med.*, 1942, **75**, 355.

[4] Daft, F. S., Sebrell, W. H., and Lillie, R. D., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 1.

[5] Green, J., and Brunschwig, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 348.

[6] Handler, P., and Dubin, I. N., *J. Nutrition*, 1946, **31**, 141.

[7] Daft, F. S., Sebrell, W. H., and Lillie, R. D., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 228.

[8] Blumberg, H., and Grady, H. G., *Arch. Path.*, 1942, **34**, 1035.

[9] Gillman, J., Gillman, T., Mandelstam, J., and Gilbert, C., *Brit. J. Exp. Path.*, 1945, **26**, 67.

[10] Himsworth, H. P., and Glynn, L. E., *Clinical Science*, 1944, **5**, 93.

[11] Himsworth, H. P., and Glynn, L. E., *Clinical Science*, 1944, **5**, 133.

[12] Glynn, L. E., Himsworth, H. P., and Neuberger, A., *Brit. J. Exp. Path.*, 1945, **26**, 326.

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1+	Few droplets in every lobule.
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Grading of Fibrosis

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received a daily oral supply of thiamin, 20 γ; riboflavin, 25 γ; pyridoxine, 20 γ; and calcium pantothenate, 100 γ. Vitamins A and D were supplied by adding 5 drops of Oleum Percomorphum[§] per kilogram of diet to the melted lard as the diet was prepared.

Diet 1. The rats weighed from 140 to 170 g and one group of 13 animals were fed Diet 1, and a second group of 10 rats received the control diet (Table I). Water and food was allowed *ad libitum*, and daily food intake was measured in 5 rats of each group who were housed in individual cages. At 85-90 days liver biopsies were performed on all of the animals in the individual cages by laparotomy under ether anesthesia. The biopsy was taken from the edge of the left lobe of the liver. This procedure was repeated on the same animals in the same manner at 155-165 days. After 190-200 days on the diets the animals were sacrificed and specimens of tissue were taken from the center of the left and right lobes of the liver and from the kidney. The remaining liver of all animals was analyzed for total lipids by a modification of the method of Outhouse and Forbes.[13] All of the tissue sections were stained with hematoxylin and eosin and in addition sections stained with Sudan III were prepared from half of the animals in each group. In a few cases Masson, reticulin, acid fast stain and Best-carmine stain were also utilized.

Diet 2. Animals fed this diet weighed from 130 to 160 g. Ten rats received diet 2 and ten animals were fed the control diet (Table 1). Food was allowed *ad libitum* and daily measurement of food intake was made on five rats of each group. No biopsies were taken on these animals. Six rats in each group were sacrificed after 150 days and the remainder on the 200th day of the experiment. Tissue sections were made as outlined under Diet 1. Lipid analyses were made on half of the animals in each group.

Diet 3. The rats used weighed between 110 and 150 g. Ten animals were fed diet 3 and 10 received the control diet. All of the animals were housed in individual cages and each animal received *only* 8 g of food per day. The exact amount of food consumed by each animal was measured daily. Five

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[13] Outhouse, E. L., and Forbes J. C., *J. Lab. and Clin. Med.*, 1939, 25, 1157.

TABLE III.
Microscopic Pathology of All Animals at Autopsy.

Group	Total animals	Duration of exp. (days)	No. of rats	No. died	No. with 1-2 + fat	No. with 3-4 + fat	No. with fibrosis	Total lipids mean + S.E.
Control diet	10	200		8	1	0	0	—
Diet I	13	200		7	0	13	11	20.50% ± 1.72
Control diet	10	150	6	1	2	0	0	8.98% ± 1.81
		200	4	1	0	1	0	9.40% ± 1.74
Diet II	10	150	6	1	0	6	3	28.19% ± 3.41
		200	4	1	0	4	3	23.14% ± 2.94
Control diet	10	60	5	0	1	0	0	—
		75	5	0	0	0	0	—
Diet III	10	60	5	0	0	4	0	—
		75	5	0	0	4	0	—

of the animals in each group were sacrificed at 60 days and the remainder on the 75th day of the study. A single piece of tissue was taken from the left lobe of the liver for microscopic examination and all sections were stained with hematoxylin-eosin.

Results. Diet 1. The animals fed Diet 1 all showed marked fatty changes in the liver on biopsy at 85-90 days (Table II). The fat was present as large droplets which almost filled the cells, pushing the nucleus and remaining cytoplasm into a narrow rim. Fibrosis had also appeared in one animal at this time.

At autopsy of the animals fed Diet 1, almost all of the livers were enlarged and all had a yellow-brown uniform color. In many cases parts of the liver, especially the lower edge of the left and median lobes, were scarred and nodular. Microscopically the end result was a trabeculation of the entire liver by strands of fibrous tissue, dividing the liver into small pseudolobules. These strands connected central veins, portal areas, and the capsule into an interlocking network. At this stage of the lesion there was less fat histologically than seen in the biopsies, and small fat droplets were more common whereas previously large fat droplets had predominated. Some of the pseudolobules were almost fat free. The progressive nature of fibrosis was apparent when the course of each animal was followed by biopsies to autopsy.

Enmeshed in the fibrous strands were scattered, isolated, large, fat-free hepatic cells, many of which were binucleated, together with masses of a light-yellow staining substance which was found to be acid fast and assumed to be ceroid. The ceroid was rarely seen other than in the fibrous areas. The findings on all animals sacrificed at 200 days or dying before that time is summarized in Table III. Only one animal fed the control diet showed any fatty change and this was of a minor degree.

The animals fed Diet I gained weight only during the initial part of the experiment and soon reached a plateau below that of the control group (Fig. 1). During the last 4 weeks of the study respiratory infections became evident in many of the animals. Of the 13 animals fed Diet 1, 7 died before the termination of the study on the 200th day. All had severe hepatic changes and 4 had pneumonia. Eight of the animals receiving the control diet also died of pneumonia during this period, but without hepatic pathology. The respiratory infections in the control group is reflected in the loss of weight of this group during the last 4 weeks of observation (Fig. 1).

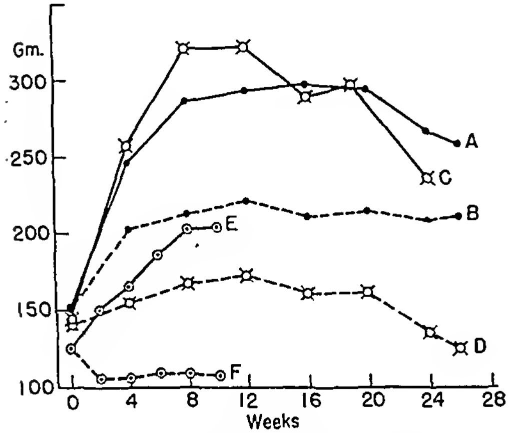

FIG. 1.

Gain in weight of rats on various diets. Curve A, control diet, and Curve B, Diet I. Curve C, control diet, and Curve D, Diet II. Curve E, control diet, and Curve F, Diet III.

Diet 2. Animals fed the low protein diet (Diet 2) also showed marked fatty changes in the liver, similar to the findings in animals fed Diet 1 (Table III). Grossly the livers of the animals fed Diet 2 were enlarged and light yellow-brown in color. In some cases the edges of the left and median lobes were nodular and firm. Microscopically the type and distribution of both the fat and fibrosis in these animals were the same as those fed the 51% fat, 16% protein diet (Diet 1). No necrosis was observed either grossly or microscopically. In four of the animals the few fat-free cells that were present contained large amounts of glycogen, a finding that was not observed in animals fed the control diet. No important differences were observed histologically between the animals fed diet 2 for 150 or 200 days, except that the lesion at 200 days was slightly more advanced.

The animals fed the low protein diet (Diet 2) gained only a slight amount of weight (Fig. 1). Two of these rats died prior to sacrifice, at 125 and 164 days respectively, both with severe hepatic changes and one also had pneumonia. Two of the animals fed the control diet died from respiratory infections during this time.

Diet 3. Animals fed this diet, which contained only 4% casein, were limited to 8 g of food per day. At autopsy, either at 60 or 75 days, the rats showed hepatic changes similar to those observed with diets 1 and 2 (Table III). Grossly the livers appeared fatty, and nodularity or necrosis was not observed. Microscopically the fatty infiltration was characterized by large fat droplets, as previously described with Diet 1. There was no fibrosis or necrosis. Large deposits of glycogen, present in those cells not laden with fat, was observed in animals fed Diet 3, and not in animals fed the control diet.

The animals fed Diet 3, limited to 8 g a day, lost weight (Fig. 1). Rats receiving the control diet, also limited to 8 g of food per day, showed a moderate gain in weight.

Discussion. Although the 3 diets used were quite different the hepatic lesions produced by them appeared to be the same. Almost all of the livers were heavily infiltrated with fat. With Diet 1, a high fat diet, and Diet 2, a low fat-low protein diet, the total lipid determinations show that the actual amount of fat in the liver was the same for both diets (Table 3). Fibrosis was not seen unless fat was also present, and the fibrosis always followed the fatty change. The fibrosis was progressive, and not the type of scarring seen following an acute injury. Minor infiltrations with fat did not result in fibrosis, in agreement with other recent studies.[14] The fibrosis was not seen in the group on the 4% casein, restricted intake diet (Diet 3), as was to be expected, since all animals were killed by the 75th day, whereas animals on the other diets did not show fibrosis until after 75 days. It then appears that the fibrosis is a result of prolonged extensive hepatic fatty change which was produced by feeding a diet high in fat

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[14] Glynn, L. E., Himsworth, H. P., and Lindan, O., *Brit. J. Exp. Path.*, 1948, 29, 1.

TABLE IV.
Comparison of the Liver Injury and Protein Intake with Data of Himsworth and Glynn.

	No. of rats	Duration, days	No. with fibrosis	No. with necrosis	Food intake g/rat/day	Protein g/rat/day	Protein g/100 g/rat/day
Diet II (6% protein, 6% fat).							
Present study	10	150-200	6	0	10.2	.614	.391
Himsworth	7	—	0	3	7.64	.458	.393
Diet III (4% protein, 6% fat) and restricted food intake.							
Present study	10	60-75	0	0	7.6	.304	.283
Himsworth	7	—	0	4	7.18	.288	.258

or by feeding a diet low in protein.

All 3 groups of animals on the control diet contained at least one animal with some increase in liver fat. In all cases the amount of fat was small and fibrosis was absent. The slight fatty change in the control animals was associated with the presence of respiratory disease.

It is difficult to compare the findings in the present study with previous ones because of the great variation in diets and techniques. However, since the methods used here were very similar to those of Himsworth and Glynn[10] a comparison can be made for each of the diets used. In both studies the 51% fat, 16% protein diet (Diet 1) produced the same fatty changes. In the present study the appearance of fibrosis was more rapid. In both cases low protein diets (Diet 2) also resulted in fatty change but Himsworth and Glynn did not find this to be followed by fibrosis as in the present study. Some of the animals fed 6% and 4% protein diets by Himsworth and Glynn developed acute hepatic necrosis, with post necrotic scarring in the surviving animals. This lesion was not observed in our animals fed the same diets (Table IV).

Himsworth and Glynn concluded from the data collected on several low protein diets that a protein intake between 200 and 500 mg per rat per day resulted in the massive necrosis. Since our 6% protein group (Diet 2) received 614 mg the failure to develop necrosis may be explained on the basis of the higher protein intake (Table IV). The protein intake, when calculated as protein per 100 g of rat weight per day, is the same in both studies. This explanation cannot hold for the failure of the 4% protein group (Diet 3) to develop necrosis, since the animals were limited to 8 g of food per day as was done by Himsworth and Glynn, and the protein intake falls well within the critical range (Table IV). Himsworth and Glynn also found that after 40 days on such a diet many of the animals became ill and died with hepatic necrosis surviving only an average of 64 days when a 4% protein diet was fed. With a similar protein intake (Diet 3) our animals did not die during the study and failed to show hepatic necrosis, either grossly or microscopically. The average calorie and protein intake for the animals on Diets 1 to 3 is summarized in Table V.

There were some minor differences in the diets used in the two studies although the percentage of constituents was the same.

TABLE V.
Average Daily Food, Calorie and Protein Intake.

Group	Food, g per rat	Calories per rat	Calories per 100 g rat	Protein, g per rat	Protein, g per 100 g rat
Control diet	13.1	54.7	20.3	2.12	.778
Diet I	7.2	46.3	22.4	1.15	.555
Control diet	14.9	62.3	23.5	2.37	.898
Diet II	10.2	42.8	27.2	0.61	.391
Control diet	7.9	33.0	19.2	1.26	.736
Diet III	7.6	31.8	29.6	0.30	.283

Himsworth and Glynn used lard in the high fat diets and arachis oil in the low fat diets, and used cod liver oil as a source of fat soluble vitamins. In the present study lard was used in all diets and the vitamins were supplied by oleum percomorphum.

Summary. 1. Groups of rats were fed diets of 16% protein and 31% fat; 6% protein and 6% fat; and 4% protein and 6% fat. These diets resulted in a fatty infiltration of the liver, and in the long term experiments this was accompanied by a diffuse, progressive, hepatic fibrosis.

2. The hepatic lesions produced by all 3 of these diets appeared to be the same type and probably had the same basic pathogenesis.

3. Hepatic necrosis was not produced by the diets used, and deaths from acute hepatic necrosis were not obtained with a low protein intake.

16874

Susceptibility of the Guinea Pig to Action of Alloxan as Compared with the Rat.*

FERMOS C. CHARALAMPOUS AND D. MARK HEGSTED.
(Introduced by Fredrick J. Stare.)

From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School, Boston.

The susceptibility to the diabetogenic as well as the general toxic action of alloxan has been studied in several species[1-9] including the human being.[10] Goldner[7] reported lesions in the islets of the guinea pig after alloxan administration but no diabetes was produced because the animals died within 24 hours. Other workers[11,12] observed changes in the blood sugar levels after alloxan injections in this species but West and Highet[13] were unable to produce alloxan diabetes in the guinea pig.

The work presented in this paper is in agreement with that of West and Highet and compares the susceptibility of the guinea pig and the rat to the action of alloxan.

Experimental. Twenty-five male guinea pigs weighing between 500 and 600 g each and kept in individual cages were divided into 5 groups. Group I consisted of 6 guinea pigs which were injected with alloxan monohydrate intravenously at various doses as shown in Table I; Group II of 7 animals partially depancreatised prior to alloxan administration. One month after the operation the animals were injected with 200 mg per kg body weight alloxan intravenously and

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* Supported in part by grants-in-aid from the American Meat Institute, Chicago, Ill., the Milbank Memorial Fund, New York City, and the Nutrition Foundation, Inc., New York City.

[1] Goldner, M. G., Gomori, G., *Endocrinology*, 1943, **33**, 297.

[2] Bailey, C. C., Bailey, O. T., *J. Am. Med. Assn.*, 1943, **122**, 1165.

[3] Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **67**, 154.

[4] Bell, F. R., *J. Comp. Path. and Therap.*, 1945, **58**, 152.

[5] Goldner, M. G., *Bull. N. Y. Ac. Med.*, 1945, **21**, 44.

[6] Banerjee, S., *Lancet*, 1944, **2**, 658.

[7] Goldner, M. G., Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 31.

[8] Mirsky, I. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 35.

[9] Langendorf, O., *Arch. Anat. and Physiol.*, 1879, **7**, 1.

[10] Conn, J. W., Hinerman, D. L., *Am. J. Path.*, 1948, **24**, 429.

[11] Sariano, M., DeFrancisis, P., *Bol. Soc. Ital. Sper.*, 1947, **23**, 307.

[12] Griffiths, M., *Aust. J. Exp. Biol. and Med. Sc.*, 1948, **26**, 339.

[13] West, E. S., Highet, D. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 60.

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Summary. 1. Groups of rats were fed diets of 16% protein and 51% fat; 6% protein and 6% fat; and 4% protein and 6% fat. These diets resulted in a fatty infiltration of the liver, and in the long term experiments this was accompanied by a diffuse, progressive, hepatic fibrosis.

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16874

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The work presented in this paper is in agreement with that of West and Highet and compares the susceptibility of the guinea pig and the rat to the action of alloxan.

Experimental. Twenty-five male guinea pigs weighing between 500 and 600 g each and kept in individual cages were divided into 5 groups. Group I consisted of 6 guinea pigs which were injected with alloxan monohydrate intravenously at various doses as shown in Table I; Group II of 7 animals partially depancreatised prior to alloxan administration. One month after the operation the animals were injected with 200 mg per kg body weight alloxan intravenously and

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[3] Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 154.

[4] Bell, F. R., *J. Comp. Path. and Therap.*, 1948, **58**, 152.

[5] Goldner, M. G., *Bull. N. Y. Ac. Med.*, 1945, **21**, 44.

[6] Banerjee, S., *Lancet*, 1944, **2**, 658.

[7] Goldner, M. G., Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 31.

[8] Mirsky, I. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 35.

[9] Langendorf, O., *Arch. Anat. and Physiol.*, 1879, **7**, 1.

[10] Conn, J. W., Hinerman, D. L., *Am. J. Path.*, 1948, **24**, 429.

[11] Sariano, M., DeFranciscis, P., *Bul. Soc. Ital. Sper.*, 1947, **23**, 307.

[12] Griffiths, M., *Aust. J. Exp. Biol. and Med. Sc.*, 1948, **26**, 339.

[13] West, E. S., Highet, D. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 60.

TABLE I.
Blood Sugar Values Following Alloxan Administration.

Treatment	Dose of Alloxan	Blood glucose in mg %					Time of sacrifice after Alloxan injection
		Hr after Alloxan inj.					
		3	8	24	48	Fasting†	
Group I	mg/kg B. wt						
24-hr fast	250	132	112	100	104		2 days
,,	225	128	120	131		110	3 ,,
,,	225	103	88				4 ,,
,,	200	150		150		100	7 ,,
,,	200	112		150		130	13 ,,
,,	200	100	115	108		120	21 ,,
Group II							
50% pancreatect. and 24-hr fast	200		120	112	135		2 ,,
,,	200	100	109	115		90	4 ,,
,,	200	120	118	115		130	8 ,,
,,	200	125	118	108		136	29 ,,
,,	200		125		112	108	16 ,,
Group III							
24-hr fast	2080*					110	2 ,,
,,	2080*					105	2 ,,
,,	2080*					115	2 ,,
,,	2080*					100	2 ,,
,,	2080*					112	2 ,,
,,	2080*					118	2 ,,
Group IV							
Vit. C deficiency and 24-hr fast	200	115	112			100	3 ,,
,,	200	125	135			113	20 ,,
,,	200		125	110		85	24 ,,
,,	200		115			90	2 ,,
Group V							
3-day fasting	200		95	110	120		2 ,,
,,	200		100	132	130		2 ,,

* Intraperitoneal injection at intervals over a 10-week period. All other groups received one intravenous injection.

† Mean value of several determinations in those cases where the time of sacrifice was later than 4 days.

were sacrificed at various intervals thereafter. Group III consisted of 6 animals which were injected repeatedly with alloxan intraperitoneally over a period of 10 weeks. A total of 2080 mg per kg was given to each guinea pig and the animals sacrificed 2 days after the last injection which was 500 mg per kg body weight. Group IV was 4 animals which were kept on a vitamin C deficient diet for a period of 36 days, prior to injection of 200 mg per kg alloxan, and Group V was 2 animals which were fasted for 3 days and then injected with 200 mg alloxan intravenously.

The intravenous injections of alloxan in all cases were made in the jugular vein after exposure of the vein with a small skin incision under ether anaesthesia. Blood samples were drawn by heart puncture and the glucose concentration was determined using the micromethod of Reinecke.[14] Urine collections were made at weekly intervals during the whole experimental period and tested for glucose using the method of Somogyi.[15]

Cytologic examination of the pancreas was made in all cases after fixing the tissue in Bouin's solution and staining by Gomori's method.[16]

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[14] Reinecke, R. M., *J.B.C.*, 1942, **143**, 351.

[15] Somogyi, M., *J. Lab. Clin. Med.*, 1941, **26**, 1220.

[16] Gomori, G., *Anat. Rec.*, 1939, **74**, 439.

TABLE II.
Effect of Age on Response of Rats to Diabetogenic and General Toxic Action of Various Doses of Alloxan Given Intravenously.

No. of rats used	Wt, g	Dose, mg/kg	Failures, %	Mild* %	Severe† %	Deaths within 2 days %
60	80-110	50	25.0	58.3	16.7	0.0
30	90-105	60	16.7	0.0	83.3	0.0
20	87-100	70	0.0	0.0	90.0	10.0
20	85-110	80	0.0	0.0	75.0	25.0
20	280-300	40	10.0	72.5	17.5	0.0
30	280-300	50	10.0	0.0	73.3	16.7
20	290-305	60	0.0	3.0	7.0	90.0

* *Under mild* are classified those young rats which drank 40-60 cc water daily and excreted 3-5 g of sugar daily in the urine, among the adult rats those that drank 60-90 cc water and excreted 5-9 g of sugar daily.

† *Under severe* are grouped those rats that drank water and excreted glucose in the urine in amounts greater than the ones stated above.

The diet consisted of stock rabbit pellets supplemented with fresh green vegetables, carrots and 8 cc of orange juice daily, the last given by pipette. The animals in Group IV received the rabbit pellets only.

Results. Table I summarizes the data with regard to treatment, size of alloxan dose, route of administration, blood sugar levels at various intervals after alloxan, and time of sacrifice in days after the last dose of alloxan was given. The blood sugar concentration at all intervals after alloxan injection ranged from 85 to 150 mg per 100 cc of blood. These values represent post-alloxan *fasting blood sugar levels* and lie in the normal range for the guinea pig. Glycosuria was never observed.

The survival of the animals following the injections of alloxan was influenced by the size of the dose, the route of administration and the previous nutritional status. A dose of 250 or 225 mg per kg body weight killed the animals within 3 days. A dose of 200 mg was well tolerated by animals fasted for 24 hours but the same dose was fatal after a 3-day fast although the blood sugar level was in the normal range. Following the administration of 400 mg per kg by the intraperitoneal route, the animals became ill and refused to eat but had recovered by the following day.

All of the animals which received over 200 mg of alloxan per kg and those which received 200 mg per kg following a 3-day fast and which were examined during the first week after injection showed gross lesions in the lungs, liver, and kidneys. The histological examination of the pancreas showed no lesions following the intravenous administration of a 200 mg per kg dose of alloxan. The animals which received the 250 and 225 mg per kg doses had lesions of various degrees of severity ranging from cloudy swelling to necrosis and disappearance of β-cells. It was observed that doses of alloxan which were fatal produced damage in a third of the islets and only 16% of the islets were classified as severely damaged.

Discussion. The data presented indicate the resistance of the guinea pig to the diabetogenic and to the general toxic action of alloxan. The maximum tolerated dose under the conditions of these studies is approximately 200 mg per kg intravenously. Slightly higher doses cause death within a few days, the blood sugar remaining normal. For comparison, data which have been collected with rats of various sizes during the past few years are shown in Table II. It will be observed that optimum dosage for the production of diabetes in the rat apparently varies with age. Young rats tolerate 60 to 70 mg per kg of body weight in agreement with the previous studies of Mann and Stare.[17] In adult animals similar results are obtained with a dosage level between 45 and 50 mg per kg of body weight.

Several other factors besides age and dosage level are known to affect the toxity and diabetogenic action of alloxan such as the concentration of the alloxan solution, fasting prior to alloxan injections, and the composition of the diet.[18-21]

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[17] Mann, G. V., Stare, F. J., *J. Lab. Clin. Med.*, 1948, 33, 1161.

The development of alloxan diabetes apparently depends upon the difference in the susceptibility of the islet tissue as compared to other body tissues. If the range is sufficiently great so that marked islet destruction with only minor damage to other tissues can be obtained, diabetes can be produced. This differential is apparently found in most mammalian species. In the guinea pig, however, the islet tissue appears no more susceptible than other tissues and the resistance of both to alloxan is much greater than is found in the rat.

Summary. The parenteral administration of various dosages of alloxan, up to those which were fatal, failed to produce diabetes in guinea pigs. Neither glycosuria nor hyperglycemia was observed. Animals deficient in ascorbic acid, partially pancreatectomised or starved prior to alloxan administration, also failed to develop diabetes, although starvation apparently increased the toxicity of alloxan.

The guinea pig is much more resistant to the diabetogenic and the toxic action of alloxan than is the rat, and the pancreatic islet cells appear no more susceptible to alloxan than the cells of several other tissues.

We are indebted to Merck and Co., Inc., Rahway, N. J., Corn Industries Research Foundation, New York City, Sheffield Farms Co., Inc., New York City, and the Wilson Laboratories, Chicago, Ill., for generous supplies of materials used in these studies.

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[18] Kass, E. H., Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 303.

[19] Houssay, B. A., Martinez, C., *Science*, 1947, **105**, 548.

[20] Lararow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 441.

[21] Banerjee, S., *Science*, 1947, **106**, 128.

16875

Anaphylactoid Shock Produced by Anti-Platelet Serum.*

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Decrease or even absence of platelets is well known during anaphylactic, peptonic and tryptic shocks. The rate of platelet disappearance is very rapid[1] and a relationship between the severity of anaphylactic shock and the amount of blood platelets removed from circulation has been emphasized.[2] The role of the platelet in the mechanism of these types of shock is not yet clear, but its interference has been verified.[3-5] Our purpose was to produce an anaphylactoid shock using a substance known as capable of inducing an experimental thrombocytopenic purpura. Substances known as producing experimental thrombocytopenia and purpura are estradiol benzoate[6] or urethane in the dog.[7] These substances act only after at least a week of daily administration and seem to be specific to dogs. These substances are not indicated to removing the platelets abruptly from the circulation with its sudden destruction and

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* Aided by a grant from dr. Guilherme Guinle.

[1] Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 376.

[2] Kopeloff, N., and Kopeloff, L. M., *J. Immunol.*, 1941, **40**, 471.

[3] Achard, C., and Aynaud, M., *C. R. Soc. Biol.*, 1909, **67**, 83.

[4] Rocha e Silva, M., *Rev. Brasil Med.*, 1945, **2**, 363.

[5] Quick, A. J., *et al.*, *Am. J. Physiol.*, 1946, **145**, 273.

[6] Arnold, O., *et al.*, *Arch. Exp. Path. and Pharmak.*, 1937, **186**, 1.

[7] Cruz, W. O., and Moussatché, H., *Blood*, 1948, **3**, 793.

TABLE I.

Animals	No.	Wt (g)	Dose of normal serum* (ml/100 g body wt)	Reactions
Guinea pigs	6	270-660	1.0	None
Rats	9	170-260	0.5	”
Rabbits	5	1100-1600	0.2	”
Dogs†	4	1600-2000	1.0	‡

* Normal rabbit serum was injected into dogs, guinea pigs and rats. Normal guinea pig serum was injected into rabbits.

† Arterial pressure was taken in another dog (6.2 kg) after injection of normal rabbit serum (0.4 ml per 100 g body weight). The blood pressure after drop (4 cm Hg) came back to normal level after a few minutes and remained normal for more than ½ hour.

‡ Soon after the injections some discomfort was observed, sometimes followed by evacuation. One dog presented more acute symptoms such as vomiting and fainting. All these abnormalities disappeared within 10 minutes and the animals remained normal for hours thereafter. A typical picture of shock was not observed.

TABLE II.

Animals	No.	Wt (g)	Dose of serum (ml/100 g body wt)	Death from shock		Animals recovered showing purpura 48 hr
				No. of animals	Time, min.	
Guinea pigs	30	128-684	.30-1.0	22	2-60	8
Dogs	7	450-7200	.10-1.0	—	—	6*
Rabbits	12	1700-3200	.11- .18	3	3-15	9
Rats	7	110-240	.10- .50	5	3- 6	2

* One puppy was sacrificed 3 hours after the injection and purpuric lesions in the intestine were very conspicuous.

purpura, but anti-platelet serum is a powerful tool for this purpose and could be applied to several species. Anti-platelet serum produces a classical picture of thrombocytopenic purpura: absence or reduction of platelets in circulation, petechiae in the intestine, skin, lungs and heart; bleeding time very prolonged, normal coagulation time, clotting retraction delayed or absent and in non-fulminating cases severe anemia through intestinal hemorrhages. According to the amount administered the animal may be killed in 24 hours or, after decrease of blood platelet volume to zero, a regeneration is processed and the number of platelets becomes normal again.

We have administered intravenously a large amount of very active anti-platelet serum trying to obtain anaphylactoid phenomena in dogs, rabbits, guinea pigs and rats. The positive results of these experiments are presented in this paper.

We used 1:16 antidog-platelet, 1:16 antiguinea pig-platelet and 1:16 antirat-platelet sera prepared in rabbits by repeated intravenous injections of platelets. The antirabbit-platelet serum was of 1:8 titre and has been prepared in guinea pigs by repeated injections in peritoneum. The platelets were obtained by fractioned centrifugation and washed several times with saline. The titration of the sera was made by the purpurigenic method.[8] 29 animals were used as control for the administration of normal serum intravenously injected in the same amounts as the higher doses in the experiments with the anti-platelet serum. The control results are shown in Table I.

Adult dogs (weight between 6.5 and 9.0 kg) have been injected with smaller doses of normal rabbit serum (one dog with 0.2 ml and 3 others with 0.4 ml per 100 g body weight). Reactions consisted in general malaise and evacuation with prompt recovery within 2 minutes after injection.

Fifty-nine animals (33 guinea pigs, 1 adult dog, 1 young dog, 5 puppies, 12 rabbits and 7 rats) have been studied. The results are summarized in Table II.

Guinea pigs. Guinea pigs were injected thru the heart. Twenty-two animals died centration of the alloxan solution, fasting prior to alloxan injections, and the composition of the diet.[18-21]

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[8] Tocantins, L. M., *Arch. Path.*, 1936, **21**, 69.

The development of alloxan diabetes apparently depends upon the difference in the susceptibility of the islet tissue as compared to other body tissues. If the range is sufficiently great so that marked islet destruction with only minor damage to other tissues can be obtained, diabetes can be produced. This differential is apparently found in most mammalian species. In the guinea pig, however, the islet tissue appears no more susceptible than other tissues and the resistance of both to alloxan is much greater than is found in the rat.

Summary. The parenteral administration of various dosages of alloxan, up to those which were fatal, failed to produce diabetes in guinea pigs. Neither glycosuria nor hyperglycemia was observed. Animals deficient in ascorbic acid, partially pancreatectomised or starved prior to alloxan administration, also failed to develop diabetes, although starvation apparently increased the toxicity of alloxan.

The guinea pig is much more resistant to the diabetogenic and the toxic action of alloxan than is the rat, and the pancreatic islet cells appear no more susceptible to alloxan than the cells of several other tissues.

We are indebted to Merck and Co., Inc., Rahway, N. J., Corn Industries Research Foundation, New York City, Sheffield Farms Co., Inc., New York City, and the Wilson Laboratories, Chicago, Ill., for generous supplies of materials used in these studies.

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[18] Kass, E. H., Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 303.

[19] Houssay, B. A., Martinez, C., *Science*, 1947, **105**, 548.

[20] Lararow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 441.

[21] Banerjee, S., *Science*, 1947, **106**, 128.

16875

*Anaphylactoid Shock Produced by Anti-Platelet Serum.**

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Decrease or even absence of platelets is well known during anaphylactic, peptonic and tryptic shocks. The rate of platelet disappearance is very rapid[1] and a relationship between the severity of anaphylactic shock and the amount of blood platelets removed from circulation has been emphasized.[2] The role of the platelet in the mechanism of these types of shock is not yet clear, but its interference has been verified.[3-5] Our purpose was to produce an anaphylactoid shock using a substance known as capable of inducing an experimental thrombocytopenic purpura. Substances known as producing experimental thrombocytopenia and purpura are estradiol benzoate[6] or urethane in the dog.[7] These substances act only after at least a week of daily administration and seem to be specific to dogs. These substances are not indicated to removing the platelets abruptly from the circulation with its sudden destruction and penic purpura and anaphylactic, tryptic or peptonic shocks.

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* Aided by a grant from dr. Guilherme Guinle.

[1] Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 376.

[2] Kopeloff, N., and Kopeloff, L. M., *J. Immunol.*, 1941, **40**, 471.

[3] Achard, C., and Aynaud, M., *C. R. Soc. Biol.*, 1909, **67**, 83.

[4] Rocha e Silva, M., *Rev. Brasil Med.*, 1945, **2**, 363.

[5] Quick, A. J., *et al.*, *Am. J. Physiol.*, 1946, **145**, 273.

[6] Arnold, O., *et al.*, *Arch. Exp. Path. and Pharmak.*, 1937, **186**, 1.

[7] Cruz, W. O., and Moussatché, H., *Blood*, 1948, **3**, 793.

The results presented in this paper show another possible means for producing anaphylactoid shock, *i.e.*, by injecting intravenously a large amount of an active anti-platelet serum. The similarity of various signs described in purpura with some verified in allergic or anaphylactic phenomena is emphasized.

Summary. Intravenous injection of large amounts of active anti-platelet serum produces in dogs, rabbits, guinea pigs and rats a picture of severe shock quite similar to anaphylactic shock. Relationship between purpura and anaphylactic phenomena is emphasized.

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Thermolability of the Bacterium-Phage Complex.

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The infection of susceptible host cells by bacterial viruses may lead to several interesting events affecting primarily the host. A number of investigators have reported an interfering effect on the growth of unrelated viruses[1-3] while others have shown that infected cells no longer divide[2,4] or were incapable of adaptative enzyme production.[5] In the course of our investigations on the effect of sodium chloride on phage formation by staphylococci at elevated temperatures,[6] it was observed that the bacterium-phage complex was much more thermolabile than either the bacterium or phage alone. The present paper is a brief account of these observations.

The K race of phage active upon *Staphylococcus aureus* (K strain) was employed throughout these experiments. Quantitative determinations of [phage] were performed by Gratia's[7] method and viable cell counts were made by plating appropriate dilutions in tryptose agar and counting the colonies developing after 24 hours incubation at 36°C. Stock bacterial cultures were prepared by harvesting the growth from tryptose agar cultures in Roux flasks after incubation at 36°C for 18-24 hours. The suspensions were chilled for one hour in an ice-water bath and were used to make mixtures in tryptose phosphate broth containing 1 x 10^8 organisms/ml. To aliquots of these preparations phage was added in concentrations varying from 5 × 10^7 to 2 × 10^8 plaque units/ml; aliquots without added phage were maintained as controls. Samples were removed at once from the experimental mixtures and controls for determination of the viable cell counts.

As soon as samples had been taken, the tubes were immersed in the ice-water bath for an additional hour. At this time aliquots were removed for plaque determinations and viable cell counts. The latter were assumed to be a measure of the uninfected bacteria since infected cells cannot reproduce and form colonies. The plaque counts were considered to indicate the number of infected cells for it is known that phage uptake by bacteria is rapid even at 5°C and that little phage remains unattached to cells after sorption has proceeded for one hour.[8]

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[1] Delbrück, M., and Luria, S. E., *Arch. Biochem.*, 1942, **1**, 111.

[2] Luria, S. E., and Delbrück, M., *Arch. Biochem.*, 1942, **1**, 207.

[3] Delbrück, M., *J. Bact.*, 1945, **50**, 151.

[4] Cohen, S. S., and Anderson, T. F., *J. Exp. Med.*, 1946, **84**, 511.

[5] Monod, J., and Wollman, E., *Ann. Inst. Pasteur*, 1947, **73**, 937.

[6] Fong, J., and Krueger, A. P., *J. Gen. Physiol.*, 1949, in press.

[7] Gratia, A., *Ann. Inst. Pasteur*, 1936, **57**, 652.

[8] Krueger, A. P., Scribner, E. J., and Brown, B. B., *J. Gen. Physiol.*, 1946, **30**, 25.

within 1 hour (70%), 9 showed the typical signs of shock with recovery and died 24 to 48 hours later with signs of purpura. Two animals died after severe thoracic hemorrhage. Only one animal presented no symptoms of shock and died 24 hours later with signs of purpura. The picture of shock obtained was identical to those classically described: respiratory discomfort followed by violent dyspnea, urination, evacuation, convulsions and death usually within 10 minutes after the injection. At autopsy very conspicuous emphysema of the lungs was always observed. This find was confirmed by microscopic examination in several animals: edema and hemorrhage were also always present.

Dogs. The dogs were injected in the jugular or saphenous vein. With the doses employed we were not able to obtain fatal outcome. A puppy sacrificed 3 hours after the onset of shock showed a dark enlarged liver and very conspicuous purpuric lesions in the intestine. The signs of shock were agitation, urination, evacuation, vomiting, tenesmus followed by elimination of mucus with small amounts of blood, prostration and pronounced drop of body temperature.

Rabbits. The rabbits were injected in the marginal vein of the ear. Fatal shock was obtained after dyspnea and convulsions. At autopsy the characteristic extreme dilation of the right side of the heart was found.

Rats. Rats were injected in the dorsal vein of the tail. Dyspnea and convulsions were the most conspicuous signs observed. At autopsy a very typical emphysema of the lungs was found.

To observe another sign usually found in anaphylactic peptonic and tryptic shocks, *i.e.*, decrease of pressure, the carotidean pressure of some animals (2 dogs, 5 rabbits and 3 guinea pigs) was registered. One adult dog (7.2 kg) was anesthetized with liquid Dial (Ciba) the pressure drops to 50% of its normal value one minute after injection and falls as low as 5% of its initial level after 8 minutes; then the pressure rises but remains at 25% of its initial value after one hour. One young dog (3 kg) showed a strikingly similar picture. Pressure changes showed a very different picture in rabbits and guinea pigs. One rabbit anesthetized with Dial and 4 rabbits and 3 guinea pigs anesthetized with urethane showed a very uniform picture: in the first 30 to 45 seconds the pressure drops 50 to 75% from the initial level and then increases very rapidly attaining hypertensive figures from 125 to 200% of normal value, about one minute after the beginning of the injection. Those high values remain for about one minute, then drop to normal or below normal levels within a few minutes.

Numerous indications of interrelationship between signs observed in purpura and some described in anaphylactic phenomena can be pointed out. Shwartzman and Arthus phenomena have been considered allergic local hemorrhage, the mechanism of which has been long ago related to a purpuric manifestation.[9,10] Petechiae in the intestine, blood in the intestinal lumen and bloody evacuation are typical of anaphylactic shock in the dog. Thrombocytopenic experimental purpura in dogs is characterized by intestinal petechiae, blood in the intestinal lumen and severe anemia through intestinal hemorrhage, all these signs being secondary to the fall of the blood platelets.[11] Numerous cases have been described of purpura in man of an allergic nature interpreted as an anaphylactoid purpura.[12] Therapeutical substances have been imputed as causing sensitization in man producing purpuric phenomena as well as allergic reactions.[13,14]

The abrupt removal of circulating platelet by a specific serum, causing sudden destruction of this blood element accompanied by anaphylaxis-like reactions, seems also to indicate a common basic mechanism of thrombocyto-

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[9] Gratia, A., and Linz, R., *Ann. Inst. Pasteur.* 1932, 50, 89.

[10] Shwartzman, G., *et al.*, *J.A.M.A.*, 1936, 107, 1946.

[11] Cruz, W. O., da Silva, E. M., and Pimenta de Mello, R., *Memórias Inst. Oswaldo Cruz*, 1945, 42, 297.

[12] Glanzmann, E., *Jahrb. f. Kinderh.*, 1920, 91, 391.

[13] Wintrobe, M. M., *Clinical Hematology.* 2nd. ed., Lea and Febiger, Philadelphia, 1947, p. 620.

[14] Watson, C. J., *et al.*, *J. Lab. and Clin. Med.*, 1947, 32, 606.

16877

Prothrombin Deficiency of the Newborn.

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(Introduced by P. György.)

From the Nutritional Service of the Department of Pediatrics and the Gastro-Intestinal Section of the Medical Clinic, School of Medicine, University of Pennsylvania, Philadelphia.

Many of the recent studies on blood coagulation have been concerned with the nature of prothrombin, its composition and the interrelationships of the various coagulation components that affect its conversion to thrombin.

Quick[1,2] at first claimed that prothrombin, as determined by the one stage method, appeared to be composed of at least two components which he designated as A and B. His original work was soon supported by other investigators[3-5] who reached similar conclusions. Seegers, Loomis, and Vanderbilt[6,7] have since prepared prothrombin that is electrophoretically homogeneous and have therefore rejected the multi-component hypothesis.

A number of recent studies, in particular those of Fantl,[8] Quick,[9] Ware,[10] Murphy,[11] Owren,[12,13] Munro[11] and MacMillan[15] are of special interest in that they present evidence of other new factors important in the conversion of prothrombin to thrombin. Ware *et al.*[16] have been able to prepare a globulin of high purity that both accelerates the prothrombin conversion and increases the yield of thrombin. Owren[13] has described a new and highly purified factor that also accelerates prothrombin conversion. It has not been settled as to whether or not this factor is identical with the substance described by Ware *et al.* MacMillan[15] has presented experiments showing that the blood of dicoumarolized patients is deficient in a factor that is different from that described by Owren.

In the present work an attempt was made to determine whether any of the previously described factors are concerned with the prolongation of prothrombin time observed in the newborn infant.

Subjects. Twelve full term infants, under 5 days of age, whose plasma exhibited a prolonged prothrombin time were selected for this study. Neither mother nor infant had received vitamin K and none showed clinical evidence of hemorrhagic phenomena. The method employed in the evaluation of the deficiency leading to the prolonged prothrombin time consisted of the addition to the infant's plasma of other plasma or serum known to contain or lack certain components. The plasma or serum added was chosen from groups of subjects as follows: 1) Plasma from 12 normal adults, (also used as controls in the prothrombin determination). Plasma from 6 normal adults stored for various lengths of time. Serum from 6 normal adults similarly stored (Series 1). 2) Plasma from 6 normal infants whose prothrombin times were within the normal range, and who had

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[1] Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

[2] Quick, A. J., *J.A.M.A.*, 1944, **124**, 734.

[3] Munro, F. L., Hart, E. R., Munro, M. P., and Walkling, A. A., *Am. J. Physiol.*, 1945, **145**, 206.

[4] Munro, F. L., and Munro, M. P., *Am. J. Physiol.*, 1947, **149**, 95.

[5] O'Neal, W. J., and Lam, C. R., *Am. J. Med. Sci.*, 1945, **210**, 181.

[6] Loomis, E. C., and Seegers, W. H., *Am. J. Physiol.*, 1947, **148**, 563.

[7] Seegers, W. H., Loomis, E. C., and Vanderbilt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

[8] Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

[9] Quick, A. J., *Am. J. Physiol.*, 1947, **151**, 63.

[10] Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **106**, 41.

[11] Murphy, R. C., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, **151**, 338.

[12] Owren, P. A., *Lancet*, 1947, **1**, 446.

[13] Owren, P. A., The Coagulation of the Blood, Oslo, 1947.

[14] Munro, M. P., and Munro, F. L., *Am. J. Physiol.*, 1947, **150**, 409.

[15] MacMillan, R. L., *Science*, 1948, **108**, 416.

[16] Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947. **169**, 231.

With the maximum number of cells infected by phage, the mixtures were next placed in a constant temperature bath and were held at 47.5°C for 40 minutes. In order to ascertain the effect of exposure to heat upon infected and uninfected bacteria, the plaque counts and viable cell counts were repeated.

Table I presents the data from two experiments of the sort just described. The bacterial controls exhibit no significant reduction in number of viable cells during the 40 minute period of exposure to 47.5°C, nor is there any measurable drop in the phage control. On the other hand, plaque counts in the experimental mixtures are reduced to 17% and 14% of the initial titres. The numbers of viable, uninfected cells in the experimental mixtures remain practically constant confirming the data secured from the bacterial controls.

Three other experiments besides the 2 included in Table I were performed with essentially identical results. The average value for thermal destruction of phage adsorbed to bacteria for the set of 5 experiments is 80%.

Normally, phage-infected cells are detectable as plaques; exposure of such complexes to very moderate heat treatment somehow inactivates the phage so that plaques are no longer produced. Normal, uninfected bacteria or phage alone are not affected by the experimental conditions employed. We have considered the possibility that the results secured might be due to the aggregation of the infected cells during the period of heating but no evidence of this mechanism was observable on direct microscopic examination. Furthermore, the effect of phage upon living susceptible cells is to increase the zeta potential[9] and thus to decrease the likelihood of agglutination.

Summary. Staphylococcal phage and the susceptible strain of *Staphylococcus aureus* are not destroyed during a 40 minute period of exposure to 47.5°C. However, when the phage is attached to bacterial cells the plaque count drops to approximately 20 percent of the initial value under like conditions.

TABLE I.
Effect of Heat on Bacterium-Phage Complex.
Treatment 60 min. at 4°C.

Sample	Viable $[B]_o$		$[P]_o$		Treatment	Viable $[B]_f$		$[P]_f$	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2		Exp. 1	Exp. 2	Exp. 1	Exp. 2
Bacterium, phage mixture	1.5×10^8	1.5×10^8	1.0×10^8	2.0×10^8		7.8×10^7	4.5×10^7	6.7×10^7	1.0×10^8
					+ 40 min. at 47.5°C	8.0×10^7	4.3×10^7	1.2×10^7	1.4×10^7
Bacteria control	1.5×10^8	1.5×10^8	—	—		1.5×10^8	1.5×10^8	—	—
					+ 40 min. at 47.5°C	1.4×10^8	1.4×10^8	—	—
Phage control	—	—	1.0×10^8	2.0×10^8		—	—	1.1×10^8	2.0×10^8
					+ 40 min. at 47.5°C	—	—	1.0×10^8	2.0×10^8

$[B]_o$ Initial number bacteria/ml.
$[B]_f$ Final number bacteria/ml.
$[P]_o$ *Initial* number plaque units/ml.
$[P]_f$ Final number plaque units/ml.

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[9] Krueger, A. P., and Mundell, J. H., PROC. SOC. EXP. BIOL. AND MED., 1937, 36, 317.

16877

Prothrombin Deficiency of the Newborn.

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(Introduced by P. György.)

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[3] Munro, F. L., Hart, E. R., Munro, M. P., and Walkling, A. A., *Am. J. Physiol.*, 1945, **145**, 206.

[4] Munro, F. L., and Munro, M. P., *Am. J. Physiol.*, 1947, **149**, 95.

[5] O'Neal, W. J., and Lam, C. R., *Am. J. Med. Sci.*, 1945, **210**, 181.

[6] Loomis, E. C., and Seegers, W. H., *Am. J. Physiol.*, 1947, **148**, 563.

[7] Seegers, W. H., Loomis, E. C., and Vanderbilt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

[8] Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

[9] Quick, A. J., *Am. J. Physiol.*, 1947, **151**, 63.

[10] Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **106**, 41.

[11] Murphy, R. C., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, **151**, 338.

[12] Owren, P. A., *Lancet*, 1947, **1**, 446.

[13] Owren, P. A., The Coagulation of the Blood, Oslo, 1947.

[14] Munro, M. P., and Munro, F. L., *Am. J. Physiol.*, 1947, **150**, 409.

[15] MacMillan, R. L., *Science*, 1948, **108**, 416.

[16] Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

TABLE I.—Series 1.
Prothrombin Time (Seconds) of Infants Plasmas and Mixtures with Other Sera or Plasmas as Indicated.

Infant	Infants deficient plasma (A)	Adult normal plasma (B)	75% A 25% B Calculated	75% A 25% B Observed	Adult stored plasma (C)	Days stored	75% A 25% C Calculated	75% A 25% C Observed	Adult serum days stored (D)	75% A 25% D
1	39.3	20.3	32	23.0*						
2	253.0	18.5	60	28.5*						
3	64.1	20.2	30	25.6						
4	37.1	19.7	27	21.5						
5	48.6	21.3	29	25.4						
6	25.7	18.9	22	18.4						
7	36.1	17.9	25	20.2	59.6	18	39	18.2	18	18.9
8	35.9	18.8	26	21.4	96.5	21	42	22.6		
9	30.7	18.8	25	21.2	83.6	50	36	18.1	50	17.4
									21	18.2
10	41.1	18.6	26	22.3	93.0	48	46	22.1	16	19.7
11	42.3	22.3	28	19.5	176.0	160	42	24.7	120	24.3
12	30.4	19.9	24.5	20.5	200.	190	30	22.3	160	27.1

* 90% A — 10% B.

TABLE II.
Prothrombin Time (Seconds) of Infants Plasmas and Mixtures with Other Plasmas as Indicated.

	Series 2				Series 3					
Infant	Infants deficient plasma (A)	Infants normal plasma (E)	75% A 25% E Calculated	75% A 25% E Observed	Adult Dicumarolized plasma (F)	75% A 25% F Calculated	75% A 25% F Observed	Adult Liver damage plasma (G)	75% A 25% G Calculated	75% A 25% G Observed
4	37.1				30.9	36	32.4			
5	48.6				53.0	48	45.7			
7	36.1	19.5	26	24.1						
8	35.9	19.0	26	23.6						
9	30.7	19.7	24	23.8	34.8	27	25.8			
10	41.1	19.6	27	24.2						
11	42.3	18.9	27	25.5	33.5	39	25.5	27.5	36	30.5
12	30.4	18.2	24	24.2	31.5	30	29.9	31.5	30	32.5

not received vitamin K (Series 2). 3) Plasma from 5 adults who had received dicoumarol for thrombotic disease but were otherwise healthy (Series 3). 4) Plasma from two adults who were suffering from liver disease (Series 3).

Technic. The prothrombin times of the various plasmas and plasma mixtures studied were determined by use of the Quick method. Details are given in a previous paper.[17] The values given represent the averages (in seconds) of at least 3 determinations. Stored sera and plasmas were kept in a refrigerator at approximately 5°C for 18 to 190 days, as indicated in Table I.

Results. It will be noted that when the plasma of infants with prolonged prothrombin times was mixed with the plasma from normal adults in the proportions recorded (Table I, Series 1) a considerable shortening of the infant's prothrombin time occurred in each case, even though only a relatively small amount of adult normal plasma had been added. When the infant's plasma was mixed with adult stored plasma or serum a similar and in some cases, a more marked decrease in prothrombin time occurred. Plasma of infants with normal prothrombin times (Table II, Series 2) also decreased the prothrombin time of the infants with abnormal plasma but not as markedly as did the adult plasma. Finally when the plasma with prolonged time was mixed with plasma of dicoumarolized adults or of those with liver disease (Table II, Series 3) a variable result was obtained, but usually the decrease in prothrombin time was slight.

Discussion. Restoration of prothrombin times in the foregoing series of experiments demonstrates that more than a deficiency of prothrombin is involved, at least insofar as this is indicated by the one stage method. Considering the mixture described in the first experiment (Table I, infant No. 1), through the use of plasma dilution curves, the total theoretical prothrombin content of the mixtures may be calculated by adding the prothrombin contributed by each of the 2 component plasmas. Because of dilution, the adult plasma (assumed to be 100% before dilution) contributed 10%. The infant (containing only 20% before dilution) could contribute no more than 18%. The final mixture would be expected to contain the sum of the contributions by each plasma, or 28%, and the expected time would be 32 seconds instead of the observed 23. This discrepancy is even more obvious in the second infant studied (Table I, No. 2). This infant's plasma, having an extremely prolonged time, could contribute practically no prothrombin, and the calculated clotting time of the mixture would be that of a 10% dilution of adult plasma or 60 seconds instead of the observed 28.5 seconds. Similar reasoning applied to the other subjects is given in the column (Table I) marked "calculated". That more than a simple deficiency of prothrombin exists is further substantiated by the demonstration that the plasma of infants, normal by the one stage test, proved to be less effective than adult plasma in restoring the prothrombin times of the deficient infants.

The evidence here presented does not support the conclusions reached by Quick,[9] according to which the plasma of these infants should be deficient in the component he designates as prothrombin A. If this were so, the plasma of patients receiving dicumarol or suffering from liver disease should restore to normal the prothrombin time of the plasma of these infants. This was not evident in the seven instances studied.

Further, deficiency of Quick's[1,9] labile factor does not seem to be the basis of the deficiency seen in the newborn, because stored plasma, in which this factor is either absent or greatly diminished, proved to be as effective as fresh plasma for restoration of the prothrombin times.

Finally, and perhaps most significant was the demonstration that stored serum, from which prothrombin, thrombin and fibrinogen would have disappeared, was also effective in bringing the prothrombin times of deficient infants to normal.

It would appear from the foregoing that the prolonged prothrombin times frequently observed in the newborn, when measured by the one stage technic, cannot be adequately explained as a deficiency of prothrombin itself or of any of the classical clotting factors. Rather it would seem that the newborn infant is unable to convert prothrombin to thrombin with the same speed and efficiency as the normal adult. This view would also explain the discrepancy observed in studies on such subjects in the past when their blood has been examined simultaneously by the one stage and two stage technics. Our experiments indicate that a factor is provided by normal plasma, aged plasma or serum that considerably enhances the conversion of prothrombin. Recently reported studies, as previously mentioned, have shown that one or more accelerator factors are involved in the normal blood clotting mechanism. At least one of these factors has been shown to be present in serum. It is our belief that deficiency of such a factor contributes to the clotting abnormalities of the newborn, heretofore attributed solely to a lack of prothrombin. Further investigation of this basic problem to confirm and amplify these findings is clearly required.

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[17] Randall, A., IV, and Randall, J. P., *Science*, 1948, **107**, 399.

Two recent papers[15,18] demonstrate that a diminution of such a factor accounts for certain clotting changes observed in dicoumarolized subjects. The observation that dicoumarolized plasma exerts little effect upon the plasma of deficient infants suggests that the changes in both plasmas may be of similar nature.

Summary. 1. Evidence is presented indicating that the prolonged prothrombin times observed in the plasmas of newborn infants, studied by the one stage technic, cannot be adequately explained as a simple deficiency of prothrombin.

2. Although there may be some diminution of prothrombin, it appears that the plasma of the newborn is deficient in a factor accelerating conversion of prothrombin to thrombin. The deficiency of this factor is remedied by the addition of small amounts of normal plasma, stored plasma or serum.

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[18] Owen, C. A., and Bollman, J. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 231.

16878

Agglutination of Sheep's Erythrocytes Sensitized with Histoplasmin.

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The need of sensitive and specific serologic tests in fungus diseases has lately arisen in cases with skin reactions to histoplasmin. Several publications[1-3] describing complement fixation tests have appeared. Recently Saslaw and Campbell[4] have reported agglutination tests using collodion particles sensitized with histoplasmin. The preparation of collodion particles, however, is very difficult. Middlebrook and Dubos[5] have reported a similar agglutination test for tuberculosis in which sheep's erythrocytes were sensitized with a specific substance extracted from tubercle bacilli.

The present report describes an agglutination test using histoplasmin-sensitized sheep's erythrocytes as antigen and sera from rabbits immunized with *Histoplasma capsulatum*.

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* Rockefeller Foundation Fellow from University of Lund, Lund, Sweden.

[1] Tennenberg, D. J., and Howell, A., *Pub. Health Rep.*, 1948, **63**, 163.

[2] Salvin, S. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 342.

[3] Saslaw, S., and Campbell, C. C., *J. Lab. and Clin. Med.*, 1948, **33**, 811.

[4] Saslaw, S., and Campbell, C. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 559.

[5] Middlebrook, G., and Dubos, R. J., *J. Exp. Med.*, 1948, **88**, 521.

TABLE I.
Determination of the Optimal Dilution of Histoplasmin (Lot H-40) for Hemagglutination. Serum Prepared in Rabbits Against the G-5 Strain of *H. capsulatum*.

Dil. of histoplasmin before mixture with red cells	Immune serum dilutions										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
1:1	—	—	—	—	—	—	—	—	—	—	—
1:2*	+	+	+	+	+	+	+	+	±	±	—
1:3	+	+	+	+	+	+	+	±	±	±	—
1:4	+	+	+	+	+	+	+	±	±	—	—
1:6	+	+	+	+	+	+	+	±	—	—	—
1:8	+	+	+	+	+	+	±	—	—	—	—
1:12	±	+	+	+	+	+	±	—	—	—	—

Controls.

Dil. of histoplasmin before mixture with red cells	Immune serum 1:10	Normal serum			Saline
		1:10	1:20	1:40	
1:1		—	—	—	—
1:2		±	±	—	—
1:3		±	—	—	—
1:4		±	—	—	—
1:6		±	—	—	—
1:8		—	—	—	—
1:12		—	—	—	—
Normal red cells	—				

* Optimal dilution.

Methods. Histoplasmin lot H-40[†] was used in this study. The red cell suspension was prepared according to the method of Middlebrook and Dubos.[5] A 0.25% suspension, however, was found to give a more sensitive test and was used instead of the 0.5% suggested in the original description.

The sera from rabbits[‡] immunized with 2 strains of *Histoplasma capsulatum* (G-5 and G-6, Army Medical Center, Washington, D. C.) were used. As controls, sera from 6 normal rabbits and from 2 rabbits infected with *Sporotrichum Schenckii* (Duke strain 2079) and finally the serum from a rabbit immunized with *Candida albicans* (Duke strain) were used.

The sensitized cells-serum mixture was incubated according to the method of Middlebrook and Dubos.[5]

The tests were read by the pattern formed by the sedimented red cells rather than by the size of the agglutinated particles, as this was found to give more accurate readings. A positive reaction (+), as described by Salk[6] in hemagglutination tests for influenza virus, showed a thin, brownish-yellow, adherent film covering the hemispherical bottom of the test tube. In a negative reaction (—), the cells rolled to the bottom and settled as a sharply demarcated red disc which was much less adherent to the wall of the tube and moved when the tube was tilted. Reactions intermediate between the homogeneous positive film and the negative disc were characteristically ring-like in form and somewhat larger than the disc in negative tubes (±). Since reactions of this degree appeared of doubtful significance they have been regarded tentatively as negative. Serum titer was defined as the highest dilution yielding a positive (+) reaction.

Results. The optimal dilution of histoplasmin lot H-40 was found to be a 1:2 dilution (Table I).

Undiluted histoplasmin hemolysed the

[†] Made available through the kindness of Dr. Arden Howell, Jr., Field Studies Branch, Division of Tuberculosis, Public Health Service, Federal Security Agency.

[‡] Generously supplied by Miss Charlotte C. Campbell, Department of Bacteriology, Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.

[6] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE II.
Results of Hemagglutination Tests with Histoplasmin (Lot H-40).

Sera of rabbits immunized with:	Serum dilution										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
H. capsulatum, G-5	+	+	+	+	+	+	+	+	±	±	—
’’ ’’ G-6	+	+	+	+	+	+	±	—	—	—	—
C. albicans, M-1*	±	±	±	—	—	—	—	—	—		
S. schenckii, S-7†	±	±	±	—	—	—	—	—	—		
’’ ’’ S-8†	±	±	±	—	—	—	—	—	—		
6 normal rabbits	—	—	—	—	—	—	—	—	—		

* Agglutination titer with *C. albicans* cells 1:2560.
† Agglutination titer with *S. schenckii* cells 1:2560.

sheep's red cells and therefore could not be used.

As shown in Table II the sera from 2 rabbits immunized with *H. capsulatum* gave titers of 1:1280 and 1:320, respectively. Sera from normal rabbits gave negative results. Sera from 2 rabbits infected with *Sporotrichum Schenckii* and from one rabbit immunized with *Candida albicans* yielded no positive reactions. These sera were shown, however, to contain antibodies against their homologous organisms by standard agglutination tests. Serum from one human case of histoplasmosis showed no antibodies.§

It would seem, therefore, from these preliminary results that this method might be useful in serologic tests for histoplasmosis.

Summary. An hemagglutination test is described using sheep's erythrocytes sensitized with histoplasmin and sera from rabbits immunized with *H. capsulatum*. Sera from normal rabbits gave negative results and no cross-reactions were found with sera containing antibodies against *S. Schenckii* and *C. albicans*.

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§ Repeated complement fixation tests of this serum, done by Miss Charlotte C. Campbell, have been similarly negative.

16879

Histological Manifestations of a Magnesium Deficiency in the Rat and Rabbit.*

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According to Tufts and Greenberg[1] two phases are observed in magnesium deficiency in rats. The first is marked by vasodilatation, hyperemia, and hyperexcitability. The second phase is characterized by the development of nutritive failure, cachexia, and kidney damage. Greenberg, Lucia and Tufts[2] showed that prolonged deprivation of magnesium eventually produces degeneration in the kidneys, histologically manifested by degenerative changes in the tubules and progressive calcification in the cortico-medullary zone, and later, in the cortex. Renal damage was also observed in calves maintained on a diet

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* This work was supported in part by a grant from the Dow Chemical Company, Freeport, Texas, through the Texas A & M Research Foundation.

[1] Tufts, E. V., and Greenberg, D. M., *J. Biol. Chem.*, 1938, **122**, 693.

[2] Greenberg, D. M., Lucia, S. P., and Tufts, E. V., *Am. J. Physiol.*, 1938, **121**, 424.

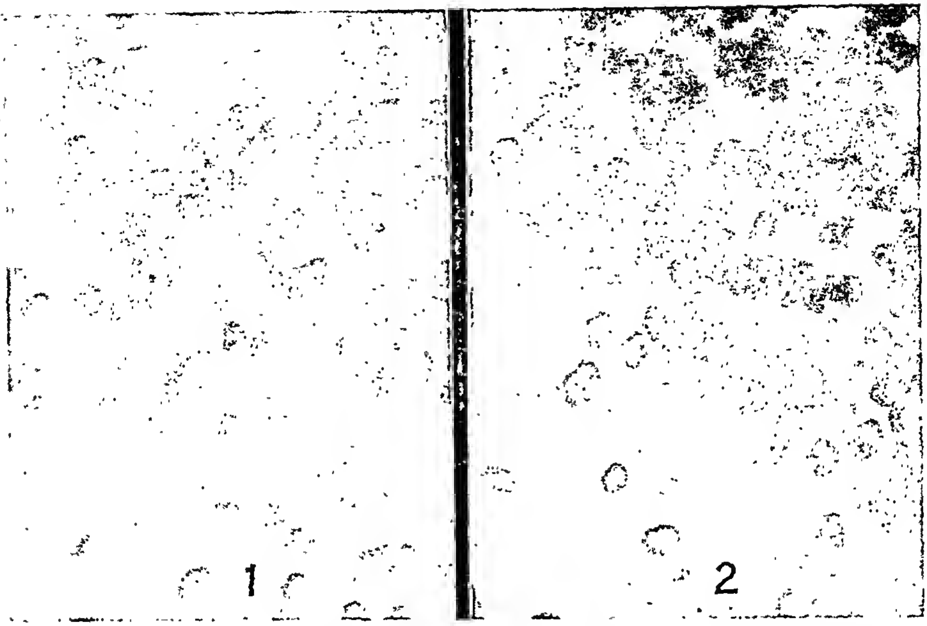

FIG. 1. Cells of Purkinje showing chromatolysis, swelling and displacement of the nucleus in a magnesium-deficient rat.
FIG. 2. Cells of Purkinje from a normal rat. (Einarson's gallocyanin chrome alum stain used in 1 and 2, × 680.)

deficient in magnesium.[3] The most consistent observation was marked proliferation of fibroblasts and fibrosis of the interstitial tissue with marked atrophy and necrosis of the parenchyma. Pathological changes in the heart due to a magnesium deficiency were reported in rats[4] and calves.[3] As Greenberg[5] has pointed out, a lack of certain B-complex vitamins has complicated the interpretations of the manifestations of a magnesium deficiency.

Bird[6] reported cerebellar lesions occurring in young chicks on a diet deficient in magnesium which were observable grossly in the severely affected chicks and only microscopically in mild cases. As far as known, no such lesions have been reported in mammals.

The work reported in this paper was designed to investigate the histological manifestations of magnesium deprivation in the cerebellum of rats and rabbits and in the kidneys and hearts of rabbits.

Experimental. Material. Cerebellar tissue was obtained from young rats that had been restricted to diets containing levels of 1 to 30 mg of magnesium per 100 g of diet for a period of 12 days. Also, cerebellar tissue was obtained from sexually mature rats that had been maintained on magnesium levels of 1 and 30 mg of magnesium per 100 g of diet for periods of 7 and 13 weeks. The dietary regimens and management of the animals has been previously reported.[7,9] In addition, cerebellar, heart, and kidney tissues were obtained from rabbits that had been started on a purified diet at weaning age and maintained on the diet which contained levels of 5 to 85 mg of magnesium per 100 g of diet for a period of 10 weeks.[8]

The brain tissue was fixed in Bouin's solution, absolute alcohol, and a solution of abso-

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[3] Moore, L. A., Hallman, E. T., and Sholl, L. B., *Arch. Path.*, 1938, **26**, 820.

[4] Greenberg, D. M., Anderson, C. E., and Tufts, E. V., *J. Biol. Chem.*, 1936, **114**, xliii.

[5] Greenberg, D. M., *Ann. Rev. Biochem.*, 1939, **8**, 269.

[6] Bird, H. F., *Poultry Sci.*, 1946, **25**, 396.

[7] Kunkel, H. O., and Pearson, P. B., *Arch. Biochem.*, 1948, **18**, 461.

[8] Kunkel, H. O., and Pearson, P. B., *J. Nutrition*, 1948, **36**, 657.

[9] Einarson, L., *Am. J. Path.*, 1932, **8**, 295.

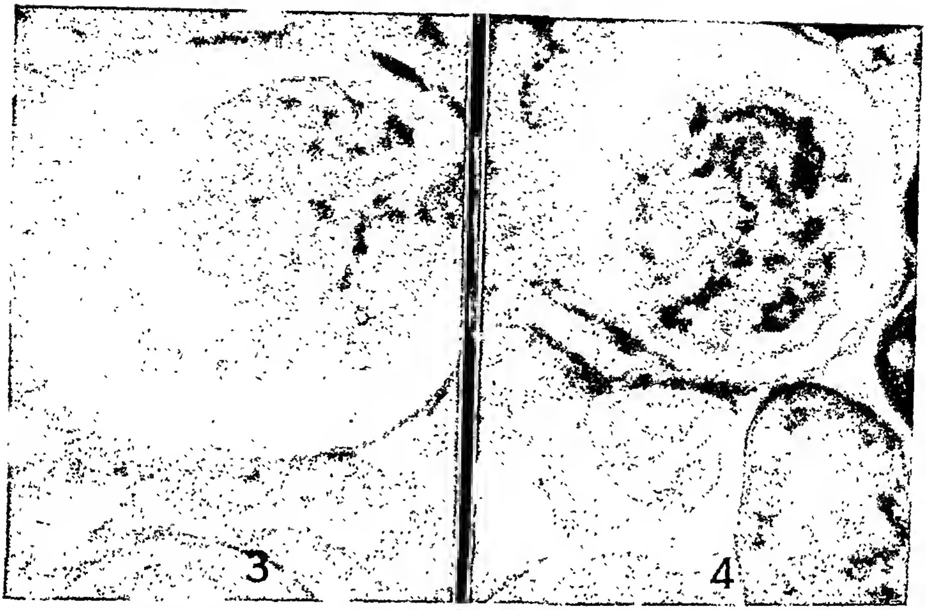

FIG. 3. Swelling and deposition of amorphous substance in the renal corpuscle of a magnesium-deficient rabbit.

FIG. 4. Normal renal corpuscle of rabbit receiving adequate level of magnesium in the diet. (Masson's trichrome stain 1 used in 3 and 4, × 680.)

lute methyl alcohol and 5% glacial acetic acid. Kidney and heart tissue were fixed in Bouin's solution and absolute alcohol. The material was imbedded in a mixture of paraffin and tissue-mat and sectioned at 7 microns. Various staining technics were used, employing silver technics, toluidine blue and erythrosin, hematoxylin and eosin, and Einarson's chrome alum gallocyanin technic.[9]

Histological observations. Histological examination of the cerebellar tissue revealed neuropathological alterations in young rats restricted to diets containing 1 mg and 5 mg of magnesium per 100 g of diet, and also in sexually mature rats restricted to a diet containing 1 mg of magnesium per 100 g of diet. At higher levels of magnesium intake, no cerebellar changes were observed.

The neuropathological changes in the rat were characterized by a degeneration of the cells of Purkinje of the cerebellum. Cytological examination revealed cells in varying degrees of chromatolysis. These cells are characterized, first, by a slight swelling, an increase in volume and a decrease in the staining property of the Nissl substance. In the second stage, there was a peripheral migration of the nucleus and an increase in vacuolation and the cell volume. The nucleus appeared to become more dense with an increase in the affinity for the stain. In some sections a number of shrunken chromatophilic cells could be observed which appeared to be the advanced stage in the degeneration of the cells of Purkinje. These observations on the neuropathological changes afford an explanation of the findings of Greenberg and Tufts[10] that protection against convulsive attacks in the magnesium-deficient rat is not afforded by the injection of magnesium salts. Also, it provides further evidence that the hyperirritability characteristic of a magnesium deficiency is not merely the result of an ion imbalance in body fluids.

Cerebellar sections from rabbits that had been restricted to diets containing 5 and 10 mg of magnesium per 100 g of diet for 10 weeks showed alteration of the cells of Purkinje. These changes were characterized by the presence of a large number of fusiform, dense and basophilic staining cells of Purkinje without a visible nucleus. Cells with the nucleus displaced to the periphery and varying degrees of chromatolysis were frequent. The swelling that was characteristic of the degenerative changes in the rat was seldom noted. The changes in the rabbit probably represent a more advanced stage of degeneration than the picture presented by the cerebellum of the magnesium deficient rat.

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[10] Greenberg, D. M., and Tufts, E. V., *Am. J. Physiol.*, 1938, **121**, 416.

Renal damage could be ascertained in sections from kidneys of rabbits maintained on a diet containing 5 mg of magnesium per 100 g of diet. This damage which involved both the renal corpuscle and tubules was not uniform throughout the kidney but was limited to certain areas of the tissue. Changes were observed in both the cortex and medulla. These degenerative changes consisted of a degeneration of the tubular epithelium and fibrosis of the corticomedullary region. Also in the renal corpuscles the capsule of Bowman remained intact as far as could be observed while the glomeruli were often displaced to the periphery by an amorphous acidophilic staining mass of material (Figures 3 and 4). Such renal corpuscles were often enlarged to as much as twice the diameter of those of the control.

X-ray examination. Sections of 2-4 $m\mu$ in thickness were removed and fixed in absolute alcohol. These sections were examined by means of an x-ray machine and plates commonly used in dental examination. Such procedure should reveal the presence of zonary calcification. No zonary calcification was observed by this technic.

Summary. Low levels of magnesium in the diet of rabbits and rats produced chromatolysis and degeneration of the cells of Purkinje of the cerebellum in the rat and rabbit. Nephrosis and fibrosis of the kidney occurs in rabbits restricted to a diet deficient in magnesium.

16880 P

Blood Oxygenation. I. The Kolff Apparatus. II. Multiple Horizontal Rotating Cylinders.[*]

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I. *The Kolff Apparatus.* In the course of construction of a perfusion apparatus capable of maintaining the circulation of an entire animal, an attempt has been made to use a method of oxygenation without an open blood-oxygen interface.

Experimental. Cellulose sausage casing was wound spirally around a horizontal revolving drum 50 cm in diameter and 50 cm long in a fashion similar to that of Kolff.[1] Casing of the following sizes was utilized: diam. 1.84 cm with 0.203 mm wall thickness, diam. 2.40 cm with 0.203 mm wall thickness, and diam. 4.16 cm with 0.406 mm wall thickness. The diffusion area was approximately 10,000 cm^2. An atmosphere of 95% O_2 and 5% CO_2 was maintained around the casing. Whipped beef blood at 37°C was introduced from a reservoir at one end of the spiral casing and collected at the other end. While the blood flowed through the casing, the drum was revolved at speeds varying between 17 and 100 r.p.m. Blood samples before and after passage through the apparatus were analyzed for oxygen content by the method of Van Slyke.[2] In some experiments the casing dipped into Ringer-Locke solutions to keep it wet. In order to utilize the mixing qualities as well as the diffusion surfaces of the spiral casing, small bubbles of oxygen were in-

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[*] Aided by a grant from the United States Public Health Service, and by a research grant from the Graduate School, University of Minnesota.

[1] Kolff, W. J., *The Artificial Kidney*, J. H. Kok, N.V. Hampen (Holland) 1946.

[2] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, 61, 523.

TABLE I.
Comparison of Oxygenators.

	Kolff	Kolff with O_2 inj. into blood*	Horizontal cylinder
cc O_2 introduced into blood per min.			
Avg	5.87		20
Max.	9.73	44	24.3
Avg vol. content, cc	200	360	70
cc O_2/min. per cc blood			
Avg	.0299		.314
Max.	.0487	.122	.406
Avg blood flow, cc/min.	150	550	250

* One experiment was done to determine the maximum oxygenation obtainable under existing conditions by this method.

troduced through a 25 gauge hypodermic needle into the blood stream as it entered the apparatus. In order to obtain a significant increase in oxygenation at least 0.5 liter of O_2 per minute had to be injected into the blood.

Results. It was found that a maximum of 9.73 cc of O_2/min. could be introduced into the blood. The blood content of the casing was 200 cc at a flow rate of 150 cc/min. Thus, a maximum of 0.0487 cc of O_2 was introduced into the blood per minute per cc of blood in the apparatus. Keeping the casing wet did not increase the oxygen uptake. Introducing bubbles of oxygen directly into the blood stream negated the theoretical advantage of no open blood-oxygen interface which this apparatus presented, and foaming resulted. At a blood flow of 550 cc/min., 44 cc of O_2 per minute was introduced into the blood per cc of blood in the casing, but 14 cc of blood was lost as foam per minute.

II. *Multiple Horizontal Cylinders.* Considering that foaming and hemolysis in an oxygenator are partly due to rapid relative motion between the blood and a surface, a slowly revolving cylinder was used upon which to film the blood.

Experimental. A metal cylinder 56 cm long and 17 cm in diameter was mounted on rollers nearly horizontally, the angle being varied from 5° to 20°. Inside this cylinder, another cylinder of approximately 12.5 cm diameter was laid. A variety of cylinders 12.5 cm diameter was used, made of fine wire screen or thin acetate plastic. The plastic cylinders were perforated by numerous holes of varying size or by rectangular openings, attempting to arrive at a design which would allow blood to film on their inner surfaces and still not foam. The 12.5 cm cylinder was weighted down by a pipe so that it would roll evenly. The surface area of this arrangement was approximately 7000 cm^2. It was revolved at various speeds, but 16 r.p.m. was found to be optimal. Blood introduced at the upper end of the incline filmed the inner surface of the metal cylinder, the outer and inner surfaces of the screen (or plastic), and the outer surface of the pipe. An atmosphere of 95% O_2 and 5% CO_2 was maintained in the apparatus.

Results. With this apparatus an average of 22 cc of O_2 could be introduced into the blood per minute. At 250 cc per minute flow, 70 cc of blood were present in the film. Thus, a maximum of 0.406 cc of O_2 was introduced into the blood per minute per cc of blood in the film. However, foaming occurred and 10 cc of blood was lost as foam per minute.

Summary. 1. Cellulose sausage casing presents too great a barrier to the diffusion of O_2 into blood to make the Kolff apparatus an efficient oxygenator. 2. Although the Kolff machine mixes blood and oxygen, the foaming which results is excessive for an oxygenator. 3. Horizontal revolving cylinders oxygenate blood efficiently enough to make their use in animal perfusion practicable, but foaming is excessive.

16881 P

Blood Oxygenation. III. The Vertical Revolving Cylinder. IV. The Vertical Revolving Cone.[*]

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III. *The Vertical Revolving Cylinder.* An apparatus of design similar to the oxygenator of Gibbon[1,2] has been studied to determine whether its efficiency could be increased.

Experimental. A plastic (Plexiglass) cylinder 38 cm long and 18 cm in diameter (2200 cm^2 area) was mounted vertically on rollers. While the cylinder revolved, a small stream of whipped beef blood was directed onto the inner surface near the top, where it formed a film, was exposed to a 95% O_2 - 5% CO_2 mixture, and was subsequently caught in a stationary cup which fitted outside of the lower edge of the cylinder. Analyses (Van Slyke[3]) for oxygen were made on blood samples before and after oxygenation.

Results. The rate of blood flow through the oxygenator changed the volume of blood in the film. At a flow of 200 cc/min. the film content was 43 cc and at 300 cc/min. the content increased to 53 cc. Varying the rate of revolution of the cylinder (100-250 r.p.m.) did not change the volume of the film. If the blood spout was lowered half the distance to the bottom of the cylinder so that half of the cylinder area was used, the film content decreased to 25 cc at 200 cc/min. and 34 cc at 300 cc/min. Thus, the upper half of the cylinder held an average of 61% of the blood in the film when the entire drum was used. Conversely, there was an average increase of 63% in the amount of blood in the film when the area of the film was doubled, keeping the diameter of the cylinder the same. There was no foaming in this apparatus.

An average of 11.53 cc and a maximum of 20.70 cc of oxygen was introduced into the blood per minute using the entire drum. Using half of the drum area an average of 9.32 cc and a maximum of 16.50 cc of oxygen was introduced per minute. Thus, an average of 81% of the total amount of oxygen intake occurred on half of the cylinder, which held 61% of the blood. Conversely, there was a 25% increase in oxygen uptake by increasing the film content 64% (utilizing the entire drum area). Expressed in terms of the amount of oxygen introduced into the blood per minute per unit volume of film, the entire cylinder introduced an average of 0.201 cc of O_2 per min. per cc of film, while the half-cylinder introduced 0.247 cc of O_2 per min. per cc of film. This represents an increase in efficiency of 23% by using half of the drum area with the same diameter. The maximum oxygen uptake observed was 0.470 cc per minute per cc film content using half-cylinder.

Varying the rate of revolution of the cylinder between 100 r.p.m. and 250 r.p.m. did not change the rate of oxygen uptake, though the film appeared to be less even at lower speeds.

IV. *The Vertical Revolving Cone.* Observations of the vertical revolving cylinder suggested that the greatest trauma to the blood occurs when it flies off the lower edge of the cylinder into the stationary cup. By using a funnel with a slope which will permit flow of blood downward to a small hole at the center, the trauma of transfer of the film blood to a non-rotating cup is minimized by virtual elimination of centrifugal factors.

Experimental. Two funnels, one 30 cm base diameter by 40 cm high, the other 20 cm base diameter by 53 cm high were re-

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[*] Aided by a grant from the United States Public Health Service, and by a research grant from the Graduate School, University of Minnesota.

[1] Gibbon, J. H., Jr., *J. Lab. and Clin. Med.*, 1939, **24**, 1192.

[2] Gibbon, J. H., Jr., and Kraul, C. W., *J. Lab. and Clin. Med.*, 1941, **26**, 1803.

[3] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

TABLE I.
Comparison of Oxygenators.

	Vertical cylinder	Half vertical cylinder	30x40 funnel	20x53 funnel	Half 20x53 funnel
cc O_2 introduced into blood per min.					
Avg	11.5	9.3	3.3	4.6	3.8
Max.	20.7	16.5	6.4	7.3	4.9
Avg vol. content, cc	48	29	42	41	26
cc O_2/min per cc blood					
Avg	.201	.247	.082	.093	.145
Max.	.390	.470	.165	.189	.195
Avg blood flow, cc/min.	250	250	200	250	250

volved in a similar manner to the cylinder, and O_2 analyses made on the blood before and after oxygenation.

Results. The efficiency of the funnel as an oxygenator was considerably less than that of the cylinder, an average of 0.10 cc and a maximum of 0.189 cc O_2 per minute per cc film content being introduced into the blood. The steeper funnel was the better oxygenator. Using 50% of the funnel area increased the efficiency as an oxygenator 56%. Varying the speed of rotation from 87-200 r.p.m. made no difference in the amount of oxygen introduced into the blood.

Summary. 1. More oxygen can be introduced into blood per unit volume of film in a vertical revolving cylinder of 18 cm diam. if segments 18 cm long are used than if the segments are 38 cm long. An increase in efficiency of 23% was obtained. 2. Speed of rotation does not, within the ranges of 100 to 250 r.p.m., affect film content or oxygenation in such a vertical revolving cylinder. 3. The vertical revolving cone does not make a good oxygenator but may conceivably be used to collect blood atraumatically from vertical cylinders.

16882

Polyethylene Plastic Needle Guides for Angiostomy.*

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A method whereby access can be gained to deep-lying vessels and chambers in the unanesthetized animal has many uses in experimental biology. London and Chlaponina[1] described a cannula for this purpose, which could be sutured to any desired large vessel and whose external end could be placed in the subcutaneous tissue. This device was used for obtaining blood samples in the course of metabolic studies. Hamilton and his coworkers[2] measured pressures in the lesser circulation through needle guides. These were constructed of metal, in two parts, and were usually seated intrapericardially in 2 operations.

The almost inert plastic, polyethylene,[3] lends itself well to the construction of needle guides that possess many advantages. These guides are prepared by blowing a suitable section of polyethylene tubing (4-6 mm o.d.) into a flattened spheroid with the aid of a

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* This work was supported by the Office of Naval Research under Project N6ori-44, Task Order XI.

[1] London, E. S., and Chlaponina, S. J., *Z. f. d. ges. exp. Med.*, 1937-38, **102**, 127.

[2] Hamilton, W. F., Woodbury, R. A., and Vogt, E., *Am. J. Physiol.*, 1939, **125**, 130.

[3] Ingraham, F. D., Alexander, E., and Matson, D. D., *N.E.J. Med.*, 1947, **236**, 362.

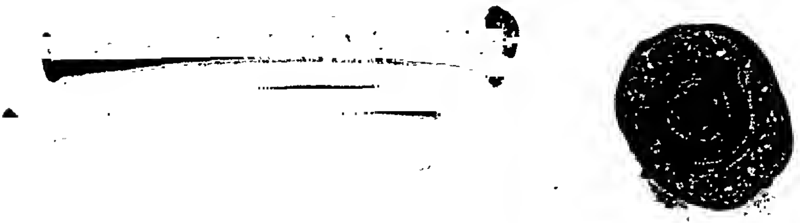

Fig. 1.

Plastic needle guide. At the left, above, is shown the guide which is sewn to the vessel or chamber. Its obturator, a segment of tubing with a cup-shaped outer end, is shown below it. At the right is an enlarged view of the foot plate showing the eyelets.

small flame. Most of the spheroid is then trimmed off leaving a curved flange which projects about 2 mm beyond the tube. A portion of the membranous segment that has been trimmed off is now faced about and sealed with a hot instrument into the flange so that its curve fits cupwise into the end of the cannula (Fig. 1). Thus the end of the tube is once again closed. This procedure is advantageous in that (1) When the needle is introduced through the guide, contact with the firm membrane at the bottom indicates that the wall of the vessel is immediately beneath. (2) After penetration, the polyethylene supports the tip of the needle and aids in keeping it from slipping too far across the lumen with possible injury of the opposite wall. (3) The membrane acts as a tampon in controlling any hemorrhage from the wall when the needle is withdrawn.

Perforations are made circumferentially through the flange in the manner illustrated in Fig. 1. These eyelets serve for suturing the guides intrapericardially to the external coats of the pulmonary artery and left auricle respectively. The attachment to the auricle is made at the posterior pericardial reflection. Sterilization of the polyethylene can be accomplished with little distortion by boiling or by soaking in an appropriate solution. Care is exercised in bringing the free ends of the cannulas to the subcutaneous position without distorting the pulmonary artery or left auricle. The cannula from the former can be brought out anteriorly along the mediastinal pleura; that from the left auricle is oriented in the interlobar fissure so as not to compress the structures of the hilum. The guides are cut to appropriate length with a scalpel. Their free ends are fixed by suture in the subcutaneous tissue of the lateral aspect of the thorax. An obturator of smaller polyethylene tubing with a cup-shaped external end may be introduced into each cannula. The chest is then closed in layers, taking care to re-expand the lungs.

The inert polyethylene in the entire operative procedure produces minimal adhesions. Healing usually is rapid and uneventful.

Blood may be obtained or pressures measured with the Hamilton manometer or other suitable means, by introducing 18-gauge "spinal" needles through the sterilized skin into the guides. If difficulty is encountered, the head of the guide can be exposed in a very short incision after anesthetizing the skin which is first displaced in a fold so made that the line of incision will later not overlie the cannula.

Summary. The construction of a polyethylene plastic angiostomy cannula is described. This cannula has many advantages for manometry of otherwise inaccessible structures in the intact animal.

16883

Solubility of Progesterone in Water and in Saline.*

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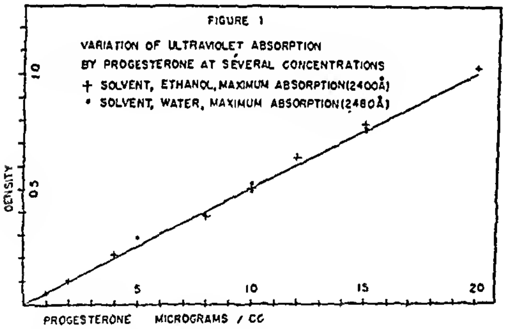
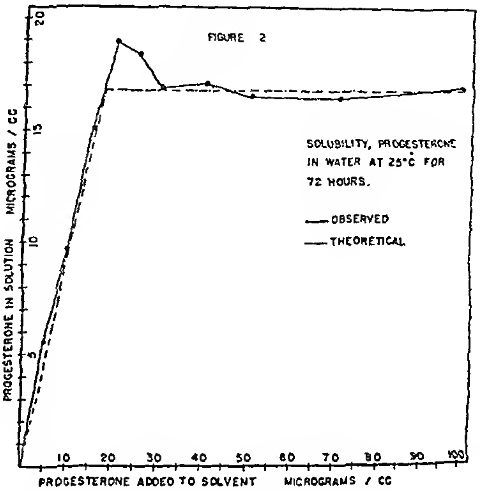
From the Department of Obstetrics and Gynecology, Washington University School of Medicine, Saint Louis, Mo.

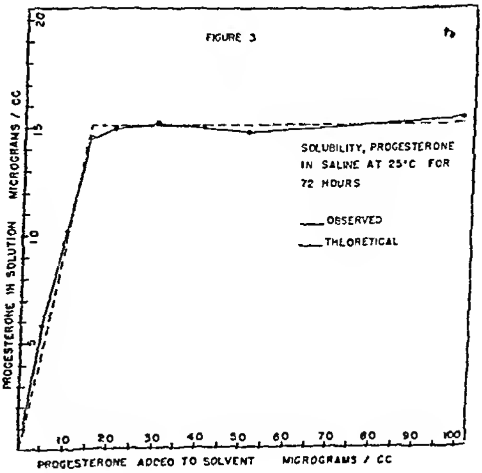
During the course of studies concerning the metabolism of progesterone, it became expedient to determine the solubility of this hormone in distilled water and in 0.9% aqueous saline. Because of the paucity of published data in this regard and its application to both *in vitro* and *in vivo* metabolic studies of progesterone, the findings are reported at this time.

The progesterone used in this study was synthetic alpha-progesterone, melting point of 128°C, and with characteristic ultraviolet absorption band at 2400 Å when dissolved in 95% ethyl alcohol. The Beckman spectrophotometer, with 1 cm square fused silica absorption cells, was used in making all ultraviolet absorption observations. Fig. 1 illustrates the relation of concentration of progesterone in solution, to ultraviolet density obtained. It is apparent that progesterone whether in aqueous or alcoholic solutions follows Beer's law.



Into chemically clean 10 ml volumetric flasks, various concentrations of progesterone in 1 ml of 95% ethanol were introduced. The concentrations varied from 50 μg/ml to 1000 μg/ml. These alcoholic solutions were evaporated to dryness on a steam bath. To the residue was added 2 ml of the intended solvent, either distilled water or aqueous NaCl (0.9%). This mixture was returned to the steam bath for 30 minutes, then removed, cooled, and diluted to a 10 cc volume with the appropriate solvent. The flasks were then placed in a closed room with mean tempera-





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* United States Public Health Fellow in Obstetrics and Gynecology.

ture of 25° C until spectrophotometric readings were made at 24, 48, 72 and 168 hour intervals on the supernatant fluid. The solubility of progesterone thus obtained is presented in Fig. 2 and Fig. 3. It should be noted that equilibrium between the progesterone and its solvent was obtained at 72 hours and that the solution concentration remained constant to 168 hours. The slight irregularity in solubility noted in Fig. 2 is thought to represent supersaturation.

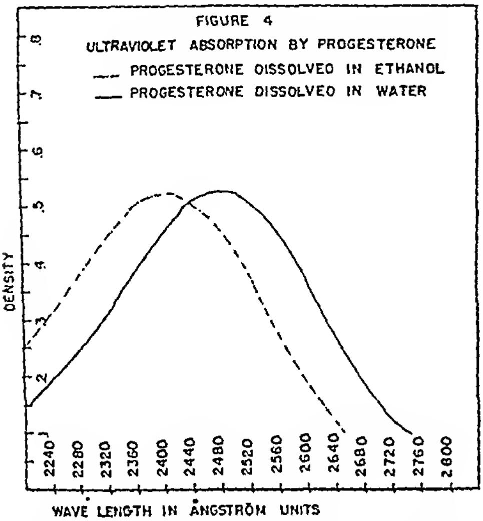
FIGURE 4

ULTRAVIOLET ABSORPTION BY PROGESTERONE

An incidental finding noted during these solubility studies was the shift of maximum ultraviolet absorption by progesterone from 2400 Å when dissolved in 95% ethanol to 2480 Å when dissolved in water. This shift occurred quite independently of the pH of the solution. Ultraviolet absorption curves are presented in Fig. 4 to illustrate this finding.

As will be noted in Fig. 2 and Fig. 3 the average solubility of progesterone in distilled water at room temperature for 72 hours was found to be 16.8 μg per ml and in 0.9% aqueous saline, 15.1 μg per ml, under identical conditions.

The solubility of progesterone in aqueous media as found in this experiment is at variance with that reported by Forbes and Hooker[1] of 6-9 μg per ml in saline. These results should not be construed as a marked disagreement since the smaller recorded solubility was based on bioassay primarily and the limited quantitative accuracy of bioassay is admitted.

Summary. The solubility of crystalline progesterone in aqueous media was determined spectrographically. Progesterone was found to have an average solubility at room temperature of 16.8 μg per ml in distilled water and 15.1 μg per ml in 0.9% aqueous saline.

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[1] Forbes, T. R., and Hooker, C. W., *Science*, 1948, **107**, 151.

16884

Cell Proliferation Accelerating and Inhibiting Substances in Normal and Cancer Blood and Urine.*

Earl R. Norris and John J. Majnarich.

From the Department of Biochemistry, University of Washington, Seattle.

The rate of cell proliferation of bone marrow cells and of normal tissue cells cultured *in vitro* is accelerated by normal blood serum and inhibited by blood serum from cases of neoplastic disease, pernicious anemia, aplastic anemia and leukemia.[1] These pathological blood sera and normal blood sera counteract the effect of each other in a manner similar to the counteracting effect of xanthopterin and antixanthopterin[2] on cell proliferation *in*

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 483.

[2] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **152**, 652.

TABLE I.

The Effect of Fractions Obtained from Normal and Pathological Urine upon the Rate of Cell Proliferation in Bone Marrow Cultures *in vitro*. Time of incubation, 7 hours. The initial concentration of the bone marrow cell suspension was: red blood cells (RBC) 8400/cmm; nucleated cells (NC) 4760/cmm; reticulocytes 11/1000 RBC.

Supplement added	Final Concentration, RBC/cmm	RBC % Increase	Final conc., NC/cmm	NC % Increase	Retic. per 1000 RBC
No supplement	10960	28	7920	66	16
5 γ xanthopterin/ml	14300	70	10600	123	32
5 γ 7-MP*/ml	4400	—48	2960	—38	0
Normal Human Urine					
Original urine	13500	61	10500	121	30
Norit filtrate	10700	27	8440	77	30
NaOH eluate	8640	2	6520	37	12
NH_3-acetone eluate	15300	82	11800	148	36
Carcinoma of the Prostate					
Original urine	13800	64	10400	118	30
Norit filtrate	10600	26	6800	43	20
NaOH eluate	4850	—42	3040	—36	4
NH_3-acetone eluate	10600	26	8600	81	22
Bronchogenic Carcinoma					
Original urine	13500	61	9080	91	28
Norit filtrate	9700	15	6850	44	20
NaOH eluate	4680	—44	2280	—52	2
NH_3-acetone eluate	10800	29	9000	89	16
Gastric Carcinoma					
Original urine	15100	92	10100	112	30
Norit filtrate	8900	6	7320	54	16
NaOH eluate	4350	—48	2000	—58	6
NH_3-acetone eluate	11700	39	8250	74	16
Hyperrenal Carcinoma					
Original urine	13600	62	8700	83	30
Norit filtrate	9050	8	6080	28	20
NaOH eluate	4800	—43	1840	—61	2
NH_3-acetone eluate	12300	46	8750	84	16
Aplastic Anemia					
Original urine	11900	42	8680	82	22
Norit filtrate	10700	27	7000	47	14
NaOH eluate	4320	—49	2200	—54	2
NH_3-acetone eluate	10600	26	7520	81	24
Carcinoma of the Rectum					
Original urine	12880	53	10000	110	26
Norit filtrate	10440	24	8520	79	20
NaOH eluate	4520	—46	2640	—45	2
NH_3-acetone eluate	12300	46	8750	84	24

* 7-MP indicates 2-amino-4-hydroxy-7-methyl pteridine.

vitro. It was suggested[1] that all blood sera contained both stimulating and inhibiting substances in varying ratios, such that normal blood serum contained a predominance of substances which would accelerate normal cell proliferation *in vitro* and blood serum from certain diseases contained a predominance of substances which would inhibit cell proliferation of normal cell suspensions and accelerate proliferation of neoplastic tissue cells.

If both accelerating and inhibiting substances are present in biological fluids, they might be separated to demonstrate their presence. For this purpose normal and pathological blood serum and urine was fractioned by adsorption on norit and elution as decribed below.

The proteins of a given volume of blood serum were precipitated with an equal volume of acetone. After mixing and filtering the precipitated proteins, the filtrate and washings were evaporated to small volume under reduced pressure to remove the acetone. The concentrated solution was diluted with distilled water to the original volume of blood serum used. The deproteinated serum extracts and urines were treated in the same manner in adsorption and elution.

An aliquot, usually 10 ml of the blood serum extract prepared as indicated above or of urine was shaken with norit and filtered. The filtrate was saved for testing on cell proliferation.

The norit was eluted with normal NaOH and filtered. The NaOH filtrate and washings were neutralized and made up to the original volume of the blood serum or urine used. The NaOH eluate was saved for testing on cell proliferation.

After elution with NaOH, the norit was eluted with ammoniacal acetone containing equal parts of 0.4M ammonium hydroxide and acetone. The filtrate and washings were concentrated under reduced pressure to small volume to remove the acetone and ammonia. The concentrated solution was diluted to the original volume of the serum or urine used for adsorption and saved for testing on cell proliferation as the NH_3 acetone eluate.

The effect of the fractions obtained was tested on bone marrow cultures *in vitro* by the technique as previously described.[2] The fractions were made up to a volume such that aliquots could be used as supplements in the bone marrow cultures equivalent to 0.1 ml of the original serum or urine for comparison. The results are shown in Tables I, III and IV. Controls are given with no supplement, xanthopterin, 2 - amino - 4 - hydroxy-7-methyl pteridine (7-MP) and Vitamin B_{14}.[3] One x 10^{-7} γ per ml of Vitamin B_{14} accelerates the rate of cell proliferation as great or greater than 5 γ per ml of xanthopterin, or Vitamin B_{14} is at least 50 million times as effective in accelerating cell proliferation in a bone marrow culture *in vitro* as xanthopterin.

Table I gives the results for fractionation of normal and pathological urines. Although neoplastic blood serum contains a predominance of inhibiting substances, the urines from cases of neoplastic disease had a predominance of substances which accelerate the rate of cell proliferation. When 0.1 ml of urine, from the neoplastic cases tested, was added to 2 ml of bone marrow suspension, the rate of cell proliferation was similar to that obtained with 0.1 ml of normal urine. The NaOH eluate contained an excess of inhibiting substances. The NaOH eluate from normal urine produced a rate of cell proliferation less than that of the control, which contained no supplement, indicating the presence of an inhibiting substance. The NaOH eluate from pathological urines all inhibited cell proliferation strongly, and had an activity similar to that of 5 γ per ml of 2-amino-4-hydroxy-7-methyl pteridine.

Table II gives the effect of fractions from neoplastic urine and other factors on the rate of cell proliferation in a suspension of cells of Brown Pearce tumor. The tumor cells were cultured by the technique previously described.[4] The effect of the pteridines, normal blood serum, and pathological blood serum on cultures of cells of neoplastic tissue *in vitro* is opposite to the effect on suspensions of normal cells.[4] Folic acid (pteroyl glutamic acid) and teropterin (pteroyl triglutamic acid) had no effect. Xanthopterin, Vitamin B_{14} and normal blood serum inhibited cell proliferation. Two-amino-4-hydroxy-7-methyl pteridine and cancer blood serum accelerated the rate of cell proliferation. The urine of broncheogenic carcinoma which accelerated

TABLE II.

Effects of Fractions from Pathological Urine Compared with Those of Other Factors on Cell Proliferation of Cells of Brown Pearce Tumor *in vitro*. The initial concentration of cells in the suspension was 18700/cmm. Time of incubation, 6 hours.

Supplement added	Final conc. of cells	% increase in cells
No supplement	21000	12
10 γ/ml folic acid	21200	13
10 γ/ml teropterin	21600	15
5 γ/ml xanthopterin	10700	—43
10 γ/ml ”	8080	—57
10^{-3} γ/ml Vit. B_{14}	10200	—45
0.1 ml normal blood serum	9950	—47
5 γ/ml 7-MP*	29000	55
10 γ/ml 7-MP*	31200	67
From Broncheogenic Carcinoma		
0.1 ml blood serum	29400	57
Original urine	13000	—30
Norit filtrate, from urine	25700	37
NaOH eluate, from urine	31000	66

* 7-MP indicates 2-amino-4-hydroxy-7-methyl pteridine.

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[3] Norris, E. R., and Majnarich, J. J., *Science*, in press.

[4] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 492.

TABLE I.

The Effect of Fractions Obtained from Normal and Pathological Urine upon the Rate of Cell Proliferation in Bone Marrow Cultures *in vitro*. Time of incubation, 7 hours. The initial concentration of the bone marrow cell suspension was: red blood cells (RBC) 8400/cmm; nucleated cells (NC) 4760/cmm; reticulocytes 11/1000 RBC.

Supplement added	Final Concentration, RBC/cmm	RBC % Increase	Final conc., NC/cmm	NC % Increase	Retic. per 1000 RBC
No supplement	10960	28	7920	66	16
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Original urine	13500	61	10500	121	30
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Original urine	13500	61	9080	91	28
Norit filtrate	9700	15	6850	44	20
NaOH eluate	4680	—44	2280	—52	2
NH_3-acetone eluate	10800	29	9000	89	16
Gastric Carcinoma					
Original urine	15100	92	10100	112	30
Norit filtrate	8900	6	7320	54	16
NaOH eluate	4350	—48	2000	—58	6
NH_3-acetone eluate	11700	39	8250	74	16
Hyperrenal Carcinoma					
Original urine	13600	62	8700	83	30
Norit filtrate	9050	8	6080	28	20
NaOH eluate	4800	—43	1840	—61	2
NH_3-acetone eluate	12300	46	8750	84	16
Aplastic Anemia					
Original urine	11900	42	8680	82	22
Norit filtrate	10700	27	7000	47	14
NaOH eluate	4320	—49	2200	—54	2
NH_3-acetone eluate	10600	26	7520	81	24
Carcinoma of the Rectum					
Original urine	12880	53	10000	110	26
Norit filtrate	10440	24	8520	79	20
NaOH eluate	4520	—46	2640	—45	2
NH_3-acetone eluate	12300	46	8750	84	24

* 7-MP indicates 2-amino-4-hydroxy-7-methyl pteridine.

vitro. It was suggested[1] that all blood sera contained both stimulating and inhibiting substances in varying ratios, such that normal blood serum contained a predominance of substances which would accelerate normal cell proliferation *in vitro* and blood serum from certain diseases contained a predominance of substances which would inhibit cell proliferation of normal cell suspensions and accelerate proliferation of neoplastic tissue cells.

If both accelerating and inhibiting substances are present in biological fluids, they might be separated to demonstrate their presence. For this purpose normal and pathological blood serum and urine was fractioned by adsorption on norit and elution as decribed below.

The proteins of a given volume of blood serum were precipitated with an equal volume of acetone. After mixing and filtering the precipitated proteins, the filtrate and washings were evaporated to small volume under reduced pressure to remove the acetone. The concentrated solution was diluted with distilled water to the original volume of blood serum used. The deproteinated serum extracts and urines were treated in the same manner in adsorption and elution.

liferation and accelerated cancer cell proliferation.

Table III gives the effect of fractions obtained from normal rat serum upon the rate of cell proliferation in bone marrow cultures *in vitro*. The normal rat serum accelerates the rate of cell proliferation. The NaOH eluate inhibits proliferation and the NH_3-acetone eluate accelerates the rate of cell proliferation more strongly than the original blood serum, when used in amounts equivalent to the original blood serum. While the fractionation was not quantitative and the substances which inhibit and accelerate the cell multiplication were not isolated, it shows definitely that both types of substances were present in the original blood serum.

TABLE V.
The Effect of Fractions from Normal Blood Serum upon the Rate of Cell Proliferation in Brown Pearce Tumor Cell Suspension, *in vitro*. Time of incubation was 7 hours. The initial cell concentration of the suspension was 9600/cmm.

Supplement added	Final conc. cells/cmm	% increase of cells
No supplement	11900	24
5 γ/ml xanthopterin	6200	—35
5 γ/ml 7-MP*	17300	80
1×10^{-6} γ/ml Vitamin B_{14}	4360	—54
1×10^{-7} γ/ml Vitamin B_{14}	7160	—25
Normal Human Blood Serum		
Original serum	6320	—34
Norit filtrate	13400	50
NaOH eluate	16000	67
NH_3-acetone eluate	7240	—24
Normal Rabbit Serum		
Original serum	7920	—12
Norit filtrate	11760	23
NaOH eluate	18600	94
NH_3-acetone eluate	6760	—29
Normal Rat Serum		
Original serum	7240	—24
Norit filtrate	13180	48
NaOH eluate	18920	97
NH_3-acetone eluate	4840	—50

* 7-MP indicates 2-amino-4-hydroxy-7-methyl pteridine.

Table IV gives the effect of fractions obtained from normal and pathological human and rabbit blood serum upon the rate of cell proliferation in bone marrow cultures *in vitro*. The supplements of serum and fractions are all equivalent to 0.1 ml of the original blood serum in 2 ml of bone marrow suspension. Normal blood sera accelerated the rate of cell proliferation. Cancer blood sera inhibited cell proliferation. The NaOH eluates all inhibited proliferation and those from the cancer blood serums inhibited more strongly than the original serum. The NH_3-acetone eluates accelerated the rate of cell proliferations. While the Norit filtrates appeared to be somewhat inhibitory, nothing definite can be said about them at this time as the adsorption on Norit may not have been complete for all factors. The results show that there was present, in all urines and blood sera studied, two types of factors which influence the rate of cell proliferation, one type which accelerates the rate of proliferation and one type which inhibits cell proliferation.

Table V gives the effect of the fractions from normal human, rabbit and rat sera shown in Tables III and IV upon the rate of proliferation of Brown Pearce tumor cells *in vitro*. The technique used was the same as that previously described.[4] The original serum, Norit filtrate and eluates were so adjusted in the experiment that each was equivalent to 0.1 ml of the original blood serum in 2 ml of tumor cell suspension. The results are opposite to those obtained on bone marrow culture, which indicates that the NaOH eluate contained factors which inhibited the normal cell proliferation and accelerated the rate of cancer cell proliferation; and the NH_3-acetone eluate contained factors which accelerated the rate of normal cell proliferation and inhibited cancer cell proliferation. The two types of factors were shown to be present in each of the original sera tested, and present in such a ratio that the net result of the original serum exhibited a predominance of factors which accelerated the rate of normal cell proliferation and inhibited cancer proliferation.

Conclusions. 1. Blood serum and urine contain two types of substances; the one which accelerates the rate of cell proliferation, and the other which inhibits the rate of cell proliferation.

2. Normal blood sera and normal and pathological urine accelerate the rate of cell proliferation in bone marrow cultures *in vitro* because of an excess of accelerating substances over inhibiting substances present.

3. Blood sera from neoplastic disease, pernicious anemia and leukemia inhibit cell pro-

TABLE III.

The Effect of Fractions Obtained from Normal Rat Serum upon the Rate of Cell Proliferation in Bone Marrow Cultures *in vitro*. The initial concentration of the bone marrow cell suspension used was: red blood cells (RBC) 6920/cmm; nucleated cells (NC) 4280/cmm; and reticulocytes 11/1000 RBC. Time of incubation, 5½ hours. All supplements were adjusted equivalent to 0.1 ml of the original serum.

Supplement added	Final Concentration, RBC/cmm	RBC % Increase	Final conc., NC/cmm	NC % Increase	Retic. per 1000 RBC
No supplement	7680	11	5360	25	18
5 γ Xanthopterin/ml	12800	85	8700	103	32
1 × 10^{-7} γ Vit. B_{14}/ml	14900	116	8400	96	34
1 × 10^{-6} γ " "	18000	160	11100	159	40
5 γ 7-MP*/ml	3820	—45	1960	—54	4
Normal Rat Blood Serum					
Original serum	14200	104	7500	75	30
Norit filtrate	8360	19	5100	19	18
NaOH eluate	4240	—39	4560	6	4
NH_3-acetone eluate	18800	173	12900	202	40

* 7-MP is used to indicate 2-amino-4-hydroxy-7-methyl pteridine.

the rate of cell proliferation of bone marrow cells *in vitro*, inhibited the proliferation of cancer cells of Brown Pearce tumor. The NaOH eluate from the cancer urine, which strongly inhibited cell proliferation in bone marrow cultures, strongly accelerated the cell proliferation in a suspension of cancer cells of Brown Pearce tumor. This showed that while in the original cancer urine there was a predominance of factors which accelerated normal cell proliferation and inhibited cancer cell proliferation a fraction was obtained as the NaOH eluate which had a predominance of substances which inhibited normal cell pro-

TABLE IV.

The Effect of Fractions Obtained from Normal and Pathological Human and Rabbit Blood Serum upon the Rate of Cell Proliferation in Bone Marrow Cultures *in vitro*. The initial concentration of the bone marrow cell suspension used was: red blood cells (RBC) 11,640/cmm; nucleated cells (NC) 4880/cmm; and reticulocytes 10/1000 RBC. All supplements added were adjusted equivalent to 0.1 ml of the original serum.

Supplement added	RBC % increase	NC % increase	Retic. per 1000 RBC
No supplement	12	24	20
5 γ xanthopterin/ml	29	77	30
5 γ 7-MP*/ml	—47	—53	4
1 × 10^{-7} γ Vitamin B_{14}/ml	60	108	40
Normal Human Blood Serum			
Original serum	26	75	36
Norit filtrate	9	30	20
NaOH eluate	—40	— 8	8
NH_3-acetone eluate	40	57	36
Serum from a Case of Bronchogenic Carcinoma			
Original serum	—35	—27	6
Norit filtrate	8	— 7	6
NaOH eluate	—79	—75	0
NH_3-acetone eluate	33	75	22
Normal Rabbit Blood Serum			
Original serum	34	81	32
Norit filtrate	7	36	18
NaOH eluate	—33	6	8
NH_3-acetone eluate	58	118	40
Serum of Rabbit Brown Pearce Tumor Blood Serum			
Original serum	—32	—28	2
Norit filtrate	1	6	10
NaOH eluate	—70	—72	0
NH_3-acetone eluate	32	81	22

* 7-MP is used to indicate 2-amino-4-hydroxy-7-methyl pteridine.

35% of normal. No changes of any significance were noted in the coagulation times.

Conclusion. It has been previously reported that methionine prolonged the bleeding and coagulation times. In this study the effects of dl-methionine on the intravenous coagulation time and prothrombin were observed in human subjects. The changes were insignificant and dl-methionine has no clinical value as an anticoagulant.

16886

Vitamin B_6 Group. XV. Urinary Excretion of Pyridoxal, Pyridoxamine, Pyridoxine, and 4-Pyridoxic Acid in Human Subjects.*

JESSE C. RABINOWITZ AND ESMOND E. SNELL.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

The earliest investigations of the excretion of vitamin B_6 by animals were made using the chlorimide reaction for analysis.[1,2] The method as then used was not specific for the determination of pyridoxine, but since the complex nature of vitamin B_6 was then unknown, all material found by this method was called *pyridoxine.* These early investigations did establish the fact that pyridoxine was rapidly absorbed from the digestive tract and rapidly cleared in the renal pathway. Although the recovery of the ingested vitamin in the rat was 50 to 70%,[1] only 10 to 20% of the dose was recovered when pyridoxine was fed to dogs or to human subjects.[2]

Subsequent refinement of the chlorimide method showed that small amounts of some substance other than pyridoxine were excreted in the urine of the dog and man after feeding a large dose of pyridoxine.[3] It was also shown that both pyridoxine and the unknown metabolite occurred in part as conjugated forms which did not react with 2, 6-dichloroquinone chlorimide, but which could be hydrolyzed with acid to give pyridoxine and the unknown metabolite.

The demonstration of the occurrence of *pseudopyridoxine* in human urine both before and after administration of pyridoxine[4] and the subsequent characterization of *pseudopyridoxine* as pyridoxal and pyridoxamine,[5] suggested the identity of the unknown metabolite of Scudi *et al.*[3] with pyridoxal or pyridoxamine.

The main metabolic product excreted after ingestion of pyridoxine was discovered by Huff and Perlzweig and identified as 4-pyridoxic acid.[6] This compound does not produce a color with the chlorimide reagent, and is inactive in promoting growth of microorganisms in vitamin B_6-free media.

Although pyridoxal and pyridoxamine are now known to be the forms of vitamin B_6 present in largest amounts in many foodstuffs and tissues,[7,8] no information is available concerning their metabolic fate. Development of a differential assay procedure for pyridoxal, pyridoxamine, and pyridoxine[8] makes such a study feasible. Results of such an investiga-

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] Scudi, J. V., Koones, H. F., and Keresztesy, J. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 118.

[2] Scudi, J. V., Unna, K., and Antopol, W., *J. Biol. Chem.*, 1940, **135**, 371.

[3] Scudi, J. V., Buhs, R. P., and Hood, D. B., *J. Biol. Chem.*, 1942, **142**, 323.

[4] Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

[5] Snell, E. E., *J.A.C.S.*, 1944, **66**, 2082.

[6] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1944, **155**, 345.

[7] Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 491.

[8] Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, 1948, **170**, 1157.

liferation in bone marrow cultures because of an excess of inhibiting substances over accelerating substances present.

4. Individual urine and blood serum specimens have been shown by adsorption on Norit and elution with NaOH and ammoniacal acetone to have both inhibiting and accelerating substances present.

16885

The Effect of Methionine on Blood Coagulation.*

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In 1936 Sterner and Medes[1] reported that cysteine and methionine prolonged both the bleeding and coagulation times for several hours when administered orally or intravenously to human subjects. They further concluded that the effect was mainly on one factor of the coagulation system, namely, prothrombin. From the data reported by these investigators one could conclude that methionine might possibly be an effective and much safer anticoagulant for clinical use than either dicoumarol or heparin. Because of these possibilities the effects of methionine on blood coagulation and quantitative prothrombin levels were studied more fully in human subjects.

In these studies dl-methionine was used. The solutions (2.5 g of methionine per 100 cc) for intravenous administration were made isotonic by the addition of sodium chloride and the pH adjusted to near neutrality by the use of sodium dibasic phosphate. The prothrombin determinations were done by the method of Quick[2] and the coagulation time by the intravenous test tube method of Lee and White.[3] Control studies were also done. All studies were done over a 6 hour period with observations at the initial 30 minute period and then each hour for the 6 hour period.

Control Studies. The coagulation times and prothrombin levels were determined in 3 persons in a fasting state over a 6 hour period. Changes were minimal. These studies were repeated in 3 persons after a routine hospital breakfast. Slight changes were noted in that the coagulation time was decreased after a period of from 4 to 5 hours, the maximum being 4½ minutes, this being within the range of experimental error.

Oral Methionine. Methionine was given to 2 individuals in 3.0 g amounts. No effect on the coagulation time was noted. No prothrombin determinations were made.

Intravenous Methionine. The individuals with normal prothrombin levels. Amounts of 1.3 g were administered to 2 subjects and no significant changes were noted in either the coagulation times or the prothrombin levels. Methionine in 2.5 g doses was given to 8 subjects, one fasting and 7 on routine hospital diet. In no instance was the coagulation time increased or the prothrombin level altered. In 4 individuals, including the fasting subject. the coagulation time was decreased, the maximum being a 10 minute decrease in one individual. The curve was a moderate exaggeration of the one obtained in the control subjects who had breakfast. Methionine in 5.0 g doses was administered to 3 subjects. No significant changes were noted in either the coagulation times or the prothrombin levels.

Intravenous Methionine. Individuals with decreased prothrombin. 2.5 g were administered to an individual with cirrhosis of the liver and observations made over a 6 hour period. The initial prothrombin level was

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* This investigation was supported by a research grant from the National Institute of Health, U. S. Public Health Service.

[1] Sterner, J. H., and Medes, G., *Am. J. Physiol.*, 1936, **117**, 92.

[2] Quick, A. J., *J.A.M.A.*, 1938, **110**, 1658.

[3] Lee, R. I., and White, P. D., 1913, **145**, 495.

TABLE II.
The Urinary Excretion of Pyridoxic Acid, Pyridoxal, Pyridoxamine and Pyridoxine.

Excretion products*	Mg excreted up to 24 hr after ingestion (a)	Mg excreted 24 hr before ingestion (b)	Mg recovered (c)	Mg excreted up to 24 hr after ingestion (d)	Mg excreted 24 hr before ingestion (e)	Mg recovered (f)
	Fed 70 mg pyridoxamine. Subject A			Subject C		
4-pyridoxic acid lactone	24.60	6.48	18.12	22.37	2.59	19.78
Pyridoxal	1.57	0.102	1.47	0.985	0.058	0.93
Pyridoxamine	1.83	0.028	1.80	1.18	0.149	1.03
Pyridoxine	0.00	0.00	0.00	0.209	0.00	0.21
Total			21.39			21.95
	Fed 82 mg pyridoxine. Subject B			Subject A		
4-pyridoxic acid lactone	28.40	1.97	26.43	25.27	3.91	21.36
Pyridoxal	1.69	0.074	1.62	1.38	0.052	1.33
Pyridoxamine	0.535	0.124	0.41	0.650	0.210	0.44
Pyridoxine	8.53	0.00	8.53	7.80	0.00	7.80
Total			36.99			30.93
	Fed 82 mg pyridoxal. Subject C			Subject B		
4-pyridoxic acid lactone	59.79	3.84	55.95	53.22	2.54	50.68
Pyridoxal	1.53	0.084	1.45	1.28	0.049	1.23
Pyridoxamine	0.021	0.083	0.00	0.241	0.097	0.14
Pyridoxine	0.039	0.00	0.04	0.132	0.00	0.13
Total			57.44			52.18

* All products are expressed in terms of the free bases. Pyridoxic acid is determined and expressed as the lactone. The molecular weight of the free bases are almost equal (pyridoxal = 167, pyridoxamine = 168, pyridoxine = 169, 4-pyridoxic acid lactone = 165). Expressed in this way, weights are directly comparable as molar quantities without significant error.

these compounds were not particularly constant. Of the 4, pyridoxic acid was quantitatively the most prominent, accounting for from 91 to 98% of the total. Of the 3 forms of vitamin B_6, pyridoxamine was usually present in highest concentrations, although pyridoxal was sometimes present in higher concentrations. Some pyridoxal was always found in the urine. The presence of pyridoxine could not be demonstrated in any of the normal urine samples; but, as noted above, this result may be due to the limitations of the method of analysis and does not constitute satisfactory evidence that pyridoxine is not excreted under normal conditions.

When pyridoxamine was fed, a significant rise in the pyridoxal as well as in the pyridoxamine content of the urine was noted; in fact, the amount of the administered pyridoxamine recovered as pyridoxal was equal to the amount recovered as pyridoxamine. The amount of pyridoxine indicated as being formed was not significant. The principal excretion product was pyridoxic acid, which accounted for 85% of the measured excretion products for subject A and 90% for subject C.

After feeding pyridoxine, the major part of the vitamin B_6 excreted was in the form of pyridoxine, but 14 to 15% of the activity appeared as pyridoxal. The pyridoxamine of the urine accounted for only 4 to 5% of the vitamin B_6 activity of the urine. In this case 71 and 69% of the excretion products measured appeared as pyridoxic acid.

When pyridoxal was fed, larger amounts of pyridoxic acid were recovered than in either of the two preceding instances. However, the tion conducted with normal human subjects are presented below.

Methods. Twenty-four hour samples of urine were collected from 3 adult male subjects and used as control samples to establish a base level for vitamin B_6 excretion by individuals on a normal diet. Individual subjects were then fed 100 mg of pyridoxine hydrochloride, pyridoxamine dihydrochloride, or pyridoxal hydrochloride in one dose with approximately 200 cc of water. Urine was collected 2, 5, 8, 12, 24, and 36 hours after ingestion of the test dose, and stored under toluene in amber bottles at 7° until analysis. Two months later the experiment was repeated using the same subjects but feeding each subject a different form of the vitamin than had been fed the first time.

The pyridoxal, pyridoxamine, and pyridoxine content of these samples was determined by the differential microbiological assay which uses *Lactobacillus casei, Streptococcus faecalis,* and *Saccharomyces carlsbergensis* as test organisms.[8] The pyridoxic acid content of the samples was determined by the fluorometric method of Huff and Perlzweig,[6] and is expressed in terms of the lactone.

Since a large part of the vitamin B_6 of natural materials is unavailable to the test microorganisms unless the samples are first autoclaved with acid,[9] control samples of urine were subjected to various conditions of acid hydrolysis to determine the optimal conditions for the liberation of vitamin B_6. The results (Table I) show that acid hydrolysis results in the liberation of increased amounts of vitamin B_6 in urine over that observed in unhydrolyzed samples. 0.055 N acid, the concentration commonly used for most samples[9,10,11] was found to be less effective than treatment with 0.11 N HCl or 0.55 N HCl. 0.11 N HCl was used instead of 0.55 N HCl in hydrolysis of the samples, since the amount of potassium chloride present in an assay tube after neutralization of samples treated with 0.55 N acid reaches approximately 135 mg per 5 cc of medium, which is close to the highest level of potassium chloride tolerated by *Saccharomyces carlsbergensis* and lactic acid bacteria.[12]

The samples collected were therefore treated in the following way prior to microbiological assay: 10 cc aliquots of each sample were autoclaved for 7 hours at 20 lb pressure in 180 cc of 0.11 N HCl. The samples were then neutralized with potassium hydroxide, diluted, and assayed.

TABLE I.
Liberation of Vitamin B_6 in Normal Urine by Acid Hydrolysis.

Treatment	Pyridoxal · HCl mγ/cc	Pyridoxamine · 2HCl mγ/cc
None	29	0
.055 N HCl*	78	133
.11 ”	93	196
.55 ”	93	210

* Autoclaved in 180 cc of acid at 20 lb pressure for 5 hr.

The limitations of the differential assay have been pointed out elsewhere,[8] especially in regard to the determination of the pyridoxine content of samples low in this form of vitamin B_6. In addition to these inherent limitations of the differential assay, it was found that the recovery of pyridoxine added to a normal urine sample was somewhat lower than could be expected from purely analytical errors.[8] Urine seemed to contain a material toxic for *Saccharomyces carlsbergensis* but not for *Streptococcus faecalis* or *Lactobacillus casei.* The presence of this toxic material results in an underestimation of whatever pyridoxine may be present in the control sample of urine, but does not affect the determination of pyridoxal or pyridoxamine in this sample. None of the results are affected in other samples, which contained much more vitamin B_6 and were therefore assayed at dilutions such that the toxic material was without effect.

Results. The distribution of the 4 compounds determined in normal urine is shown in columns *b* and *c*, Table II. The levels of

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[9] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem.*, Anal. Ed., 1943, **15**, 141.

[10] Rubin, S. J., Scheiner, J., and Hirschberg, E., *J. Biol. Chem.*, 1947, **167**, 599.

[11] Rabinowitz, J. C., and Snell, E. E., *Anal. Chem.*, 1947, **19**, 277.

[12] MacLeod, R. A., and Snell, E. E., *J. Biol. Chem.*, 1948, **176**, 39.

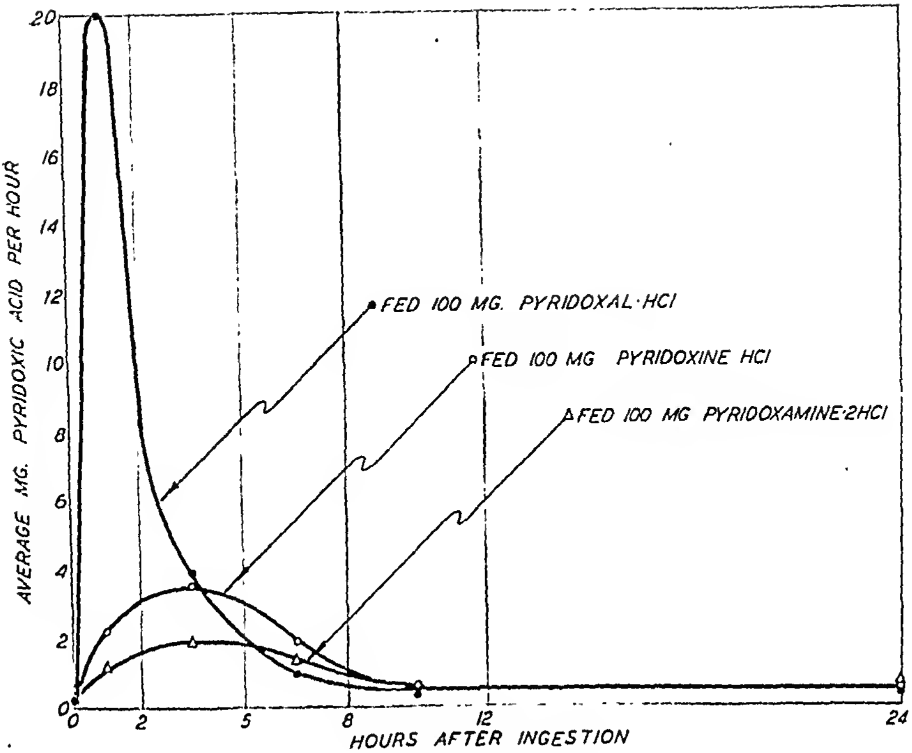

FIG. 2.

The excretion of pyridoxic acid (expressed as 4-pyridoxic acid lactone) following ingestion of various forms of vitamin B_6.

the fact that excretion levels rapidly returned to basal levels indicates that no great storage occurred. A third explanation for these low recoveries would be that still unidentified metabolic products which are without vitamin activity are formed from vitamin B_6 and excreted. From the considerations outlined above, this hypothesis seems most likely. It finds additional experimental support in the observation of Scudi, *et al.*[2] that following injection of 50 mg of pyridoxine hydrochloride in dogs, only 18% of this dose could be recovered in the urine. Yet, according to Huff and Perlzweig,[15] dogs do not excrete 4-pyridoxic acid. Unidentified excretion products, or complete oxidation within the body of considerable portions of the vitamin are thus indicated.

Pyridoxal is the only form of the vitamin which appears in the urine in greatly increased amounts as the result of feeding all 3 compounds. This further emphasizes the position of central importance indicated for this compound by direct assay of animal tissues,[8] by its universal availability to all microorganisms tested,[16] and by its occurrence in the only known catalytically active form of vitamin B_6, pyridoxal phosphate.[17]

Finally, it should perhaps be emphasized that the metabolic changes described above are those which occur when large amounts of vitamin B_6 are superimposed on a normal diet which already supplies the relatively small amounts of vitamin B_6 presumably required by man. Whether the small quantities normally ingested are metabolized in the same manner cannot be decided from these data.

Summary. The known metabolic products

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[15] Huff, J. W., and Perlzweig, W. A., *Science*, 1944, 100, 15.

[16] Snell, E. E., and Rannefeld, A. N., *J. Biol. Chem.*, 1945, 157, 475.

[17] Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1948, 161, 311.

total amount of vitamin B_6 in the urine was somewhat lower. 82 and 98% of this was pyridoxal.

Fig. 1 illustrates the fact that after feeding each of the 3 forms of vitamin B_6, a large increase in the level of the form fed occurs within 2 to 5 hours after administration of the dose, and that this level returns to the normal value within 8 hours. Similar results, not shown in this graph, were also obtained in connection with the excretion of the forms of the vitamin other than the one fed. The pyridoxic acid levels of the urine, shown in Fig. 2, increase in a much more pronounced manner, and levels did not return to normal until 12 hours after ingestion of the dose.

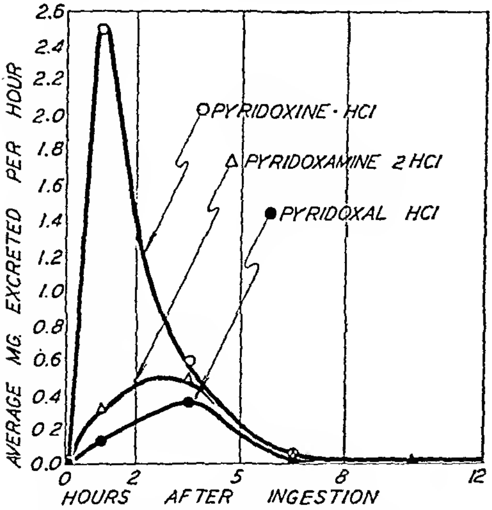

FIG. 1.

The Excretion of Vitamin B_6. The total amount of the indicated form of the vitamin found in the urine samples is plotted against the average time interval, after ingestion of the dose, during which the sample was collected.

○ Fed 100 mg of pyridoxine HCl.
△ Fed 100 mg of pyridoxamine 2HCl.
● Fed 100 mg of pyridoxal HCl.

The levels of the forms of the vitamin other than the one fed are not shown.

The amount of the test dose recovered was characteristic of the form of the vitamin fed, judging from the agreement of the results obtained in the two experiments, in which different subjects were fed the same compound. The highest recovery of the test dose, 70% and 64%, was obtained after feeding pyridoxal. The amount of pyridoxine recovered as the 4 products measured was 45 and 38% while the smallest recovery, 31 and 31%, was obtained after feeding pyridoxamine. These results may be inferred from Fig. 2, since the major component of the excretion products is pyridoxic acid.

Discussion. The above comparison of the amount of the test dose recovered as excretion products is of interest, since with pyridoxine and pyridoxamine, less than half of the administered dose was recovered. With pyridoxal, the most highly oxidized form of the vitamin, the recovery was higher because of the increased excretion of 4-pyridoxic acid which resulted when this form was fed.

A number of explanations for these low recoveries might be postulated. It is possible, for example, that the absorption of the various forms of the vitamin from the tract is incomplete. This explanation, however, is not in accord with the observations of Scudi, *et al.*,[2] who showed with both dogs and human beings that the total urinary excretion of vitamin B_6 following administration of large doses of pyridoxine (50 to 100 mg) was closely similar whether the vitamin was given orally or by intravenous injection. This indicates rather conclusively that absorption of test doses of this size from the tract is complete. The amounts of "pyridoxine" (total chlorimide reacting substances) which they found excreted following ingestion of 100 mg of pyridoxine hydrochloride approximate closely the amount of vitamin B_6 (pyridoxal, pyridoxamine and pyridoxine) found in the urine of our subjects. When limited amounts of the three forms of the vitamin are fed to deficient rats, chicks, or dogs apart from the diet, they show equal growth-promoting activities,[13,14] again indicating equal absorption.

A second possible explanation for low recoveries would be that the ingested dose was being stored in the tissues. However, the subjects used were ingesting normal diets and thus were presumably not deficient in vitamin B_6. Consequently, no great storage of the ingested vitamin would be expected, and

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[13] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **165**, 55.

[14] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 284.

of dietary protein deficiency and to attempt to relate this process to the loss of protein from various body compartments.

Materials and methods. The subjects of the experiments were young adult male albino rats (Sprague-Dawley). On arrival at the laboratory the animals were housed 7 to a cage and fed Wayne Dog Chow until they had reached a weight between 200 and 220 g. They were then started on the low protein regime. The detailed composition of the low protein diet (3E) has been described elsewhere.[6] The ration used in the present experiment contained corn oil as the fat. The content of all dietary essentials in this diet is sufficient to support normal growth when an adequate protein supplement is added. For the first 30 days of the depletion period the animals were offered 15 g of the ration each day. After 30 days the quantity was increased to 20 g per animal per day. Diet consumption is practically complete with this diet until more than 25% of the body weight has been lost after which diet intake slowly falls. The most depleted animals in this experiment never ate less than 72% (14.4 g) of their diet on the average.

In this study groups of 5 rats having comparable weights prior to depletion were selected from larger lots which had been on the depletion regime for 0, 17, 32, 43, 56, and 100 days. At the start of the final period of the experiment the animals were placed in individual cages and fed the same low protein diet for the last 11 days. On the day they were placed in the individual cages each animal was given by tail vein an injection of one ml of a 0.25% suspension of 5 times washed sheep's erythrocytes, and one ml of a heat killed vaccine of Friedlander's bacillus containing 1.6 billion organisms per milliliter. Six days after the injection of antigen, blood volumes, hematocrits, hemoglobins, serum protein concentrations and agglutinin and hemolysin titers were determined.[7] Five days following this the animals were sacrificed. The

TABLE I.* Protein Content Several Body Compartments and Rate of Antibody Formation in Rats on Low Protein Diet for Various Periods of Time.

Depletion to sacrifice, days	No. of animals	Live wt at sacrifice, g	Carcass protein, g	Total circulating serum protein, mg	Liver protein, g	Total circulating hemoglobin, mg	Depletion at time of inj., days	Agglutinin titers	Hemolysis titer
0	5	206 ± 1.6	32.1 ± .58		1.34 ± .063				
11	5	177 ± 2.1	30.8 ± .33	514 ± 19	1.07 ± .038	2370 ± 36	0	3840 ± 0	7680 ± 0
28	5	171 ± 2.2	31.0 ± 0.22	410 ± 7	1.25 ± .067	1900 ± 94	17	2200 ± 300	6700 ± 940
43	5	169 ± 3.7	30.3 ± 0.60	390 ± 10	0.91 ± .047	1410 ± 79	32	1280 ± 220	1920 ± 0
54	5	161 ± 1.9	30.7 ± 0.75	380 ± 6	0.84 ± .042	1490 ± 61	43	960 ± 0	1920 ± 390
67	5	147 ± 1.4	29.1 ± 0.60	341 ± 12	0.73 ± .021	1290 ± 61	56	320 ± 90	1280 ± 370
111	4	129 ± 4.6	23.2 ± 1.00	266 ± 15	0.70 ± .041	1150 ± 69	100	18 ± 3	340 ± 70

* Values are given as means and standard errors for groups of animals.

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[6] Wissler, R. W., Woolridge, R. L., Steffee, C. H., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

[7] Benditt, E. P., Straube, R. L., and Humphreys, E. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 189.

of vitamin B_6—pyridoxal, pyridoxamine, pyridoxine and pyridoxic acid—were measured in normal human urine and in the urine of human subjects each fed one of the 3 forms of the vitamin.

The chief product found, regardless of the form fed, was pyridoxic acid. Pyridoxal gave rise to significantly higher amounts of this product than did pyridoxine or pyridoxamine. No evidence could be obtained showing the conversion of pyridoxal or pyridoxamine to pyridoxine. When pyridoxal or pyridoxine was fed, the chief form in which the vitamin occurred in the urine was the form fed. However, when pyridoxamine was fed both pyridoxal and pyridoxamine were excreted in approximately equal amounts. Ingestion of pyridoxine also greatly increased the amount of pyridoxal and pyridoxamine excreted.

The excretion of all products was very rapid. The largest amounts of each of the compounds were found in samples collected 2 and 5 hours after ingestion of the dose. The levels of pyridoxic acid returned to normal values after 12 hours, while the vitamin levels had returned to normal within 8 hours. The amount of the dose recovered varied with the form fed. The highest recovery, 70%, was obtained when pyridoxal was fed; 45% of the pyridoxine was recovered, while only 31% of the pyridoxamine could be recovered. Together with published data which indicate that complete absorption of large doses of vitamin B_6 occurs, these findings suggest that a large proportion of the vitamin B_6 was converted to products still unknown.

16887

Loss of Body Protein and Antibody Production by Rats on Low Protein Diets.*

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(Introduced by Paul R. Cannon.)
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Previous studies in this laboratory have demonstrated that prolonged severe deficiency of protein without other dietary restriction results in a reduced capacity of the rat to produce antibodies to sheep erythrocytes,[1] Friedlander's bacillus,[2] pneumococci,[3] and most recently a parasitic nematode.[4] Concomitant with the reduction of antibody production there was shown to be a reduction in the capacity to form leukocytes of both the granulocytic and lymphocytic series.[5] Associated with these phenomena there is a reduction in the resistance of animals to infection with virulent organisms.[3] Furthermore this reduction in resistance was shown to be due largely to the inability of the animals to fabricate antibodies since the depleted animals, when passively immunized, survive the infection as well as the normally nourished controls. Having established the fact that protein depletion of long duration reduces the rate of antibody formation and the resistance to infection it then becomes of interest to investigate the rate of decay of the antibody forming capacity with time under conditions

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* The research which this paper reports was undertaken in cooperation with the Navy Department, Office of Naval Research. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

The work has been aided, also, by the National Livestock and Meat Board and the Douglas Smith Foundation for Medical Research of The University of Chicago.

[1] Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. Surg.*, 1944, **120**, 514.

[2] Woolridge, R. L., unpublished observations.

[3] Wissler, R. W., *J. Infect. Dis.*, 1947, **80**, 264.

[4] Woolridge, R. L., unpublished observations.

[5] Asirvadham, M., *J. Infect. Dis.*, 1948, **83**, 87.

necessary to exhaust the "protein reserves" of their animals and reduce the rate of plasma protein fabrication significantly.

Since antibody formation appears to be influenced by dietary protein intake it seems likely in general that anything which interferes substantially with protein metabolism such as caloric inadequacy[8] for a sufficient length of time will also impair antibody formation.

One further point is apparent in the data of this experiment. The protein *content* of any body compartment is an insensitive indicator of the rate of turnover or fabrication of protein in the compartment. In Fig. 1 it can be seen that when the total circulating serum protein fell to about 50% of its initial value the rate of antibody production has fallen to 1% or less of its initial value. From previous experiments[12] we know that the circulating quantity of that portion of the globulin containing the antibody is reduced only to an equal or lesser extent than the total protein, *i.e.* 50% or less. Practically this means that the measurement of such quantities as serum protein concentration, total circulating serum protein or any one of its components may give little if any insight into the *rate* of production or turnover of the protein.

Summary. The present experiment investigated the rate of loss of the capacity to produce antibody in comparison with the rate of loss of protein from body compartments including carcass, liver and plasma. Young adult male albino rats were tested at intervals from 0 to 100 days after initiation of the low protein diet for agglutinin and hemolysin production. Blood volumes, hemoglobin, serum protein, liver and carcass protein were determined. It was found that all protein compartments and the rate of antibody production fell at approximately linear percentage rates per day, the antibody production falling much faster than the rest. Statistically significant depression of the antibody titers was not reached till the 30th day with hemolysins and the 17th day with the agglutinins.

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[12] Benditt, E. P., unpublished observations.

16888

Chemotherapy of *Trichomonas foetus* Infections in Rabbits.*

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No satisfactory therapeutic agent has been established for the successful treatment of bovine trichomoniasis. *In vivo* studies in laboratory animals of the trichomonacidal properties of various chemicals may offer useful information for future treatment trials in cattle. The purpose of this paper is to present the results of a study on the effect of 58 compounds on *Trichomonas foetus* in the vagina of rabbits. The majority of the compounds were selected from 350 chemicals previously tested *in vitro* by Morgan and Campbell.[1]

Witte[2] apparently was the first to successfully infect rabbits with *T. foetus* by vaginal

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† Wellcome Research Fellow of the Animal Health Trust (from the Ministry of Agriculture and Fisheries Laboratories, Weybridge, England).

[1] Morgan, B. B., and Campbell, H. M., *Am. J. Vet. Res.*, 1946, 7, 45.

[2] Witte, J., *Arch. wiss. prakt. Tierheilk.*, 1933, 66, 333.

carcasses and livers were analyzed for fat, water and protein by methods which have been described.[8] Sections were made of the livers and these in conjunction with the detailed liver analyses are the subject of another report.[9]

Observations. The observations are summarized in Table I. In Fig. 1 are plotted the data for carcass protein, liver protein, total circulating serum protein, and the antibody titers (agglutinins and hemolysins). They are plotted on a semilogarithmic scale with time on the linear axis. Such a chart allows one to compare the relative rates of decline of these various factors by comparing the slopes of the curves.

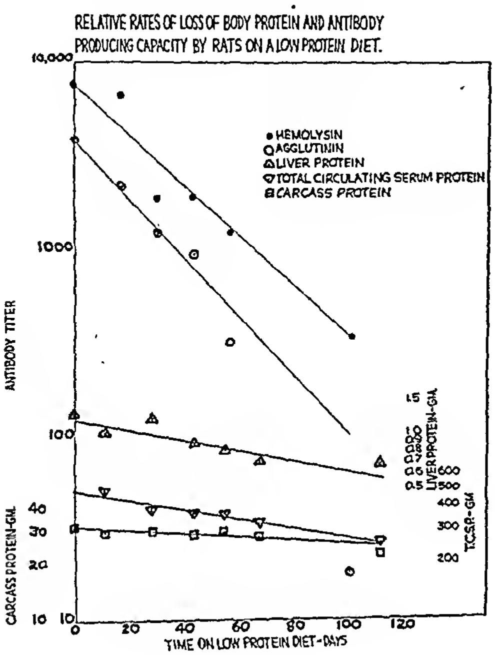

FIG. 1.

To a reasonable first approximation the loss of protein from the carcass, liver, serum and erythrocytes occurs at a constant percentage rate. The same is true for the loss of the capacity to form antibody. The daily percentage rates of decline as estimated from these curves are: Carcass, 0.3; total circulating serum protein 0.6; liver protein, 0.7; rates of antibody production, antibacterial agglutinins), 3.7; and (anti-sheep erythrocyte hemolysin), 3.0. The rate of loss of hemoglobin is approximately the same as for serum protein. Liver and plasma proteins decline at approximately equal rates, and approximately twice as fast as the carcass protein. The antibody forming capacity declines 10-13 times as fast as the carcass protein content.

Statistical analysis shows that the decrease in hemolysin titer is not significant until after 30 days following initiation of the low protein diet. The agglutinin titers appear to be just significantly diminished ($p < .05$) by the 17th day of the depletion regime.

Discussion. Recently Metcoff and co-workers[10] have questioned the role of dietary protein deficiency in impairment of antibody formation. These investigators injected young rats with *S. typhimurium* 6 days after initiation of the low protein diet. As might be expected, they could find no difference in antibody titers up to 28 days from the start of the protein deficiency. On the 28th day they injected typhoid "O" antigen and 14 days later removed blood for titers in an attempt to demonstrate an "anamnestic" response. From their experience these authors conclude "that dietary protein deficiency does not seriously impair the mechanism for protective antibody formation in the rat."

The present observations demonstrate clearly that severe restriction of the dietary protein intake, despite the presence of an adequate supply of other essential nutrients causes marked impairment of antibody production. Further they demonstrate that the capacity to form antibody decreases with time on the low protein diet but the decrease does not become appreciable with some antigens until several weeks or more have elapsed. Whipple and his co-workers[11] early recognized that many weeks of protein depletion were necessary to exhaust the "protein reserves" of their animals and reduce the rate of plasma protein fabrication significantly.

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[8] Benditt, E. P., Humphreys, E. M., Wissler, R. W., Steffee, C. H., Frazier, L. E., and Cannon, P. R., *J. Lab. and Clin. Med.*, 1948, **33**, 257.

[9] Jaffe, E. R., Humphreys, E. M., Benditt, E. P., and Wissler, R. W., *Arch. Path.*, in press.

[10] Metcoff, J., Darling, D. B., Scanlon, M. H., and Stare, F. J., *J. Lab. and Clin. Med.*, 1948, **33**, 47.

[11] Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

Since antibody formation appears to be influenced by dietary protein intake it seems likely in general that anything which interferes substantially with protein metabolism such as caloric inadequacy[8] for a sufficient length of time will also impair antibody formation.

One further point is apparent in the data of this experiment. The protein *content* of any body compartment is an insensitive indicator of the rate of turnover or fabrication of protein in the compartment. In Fig. 1 it can be seen that when the total circulating serum protein fell to about 50% of its initial value the rate of antibody production has fallen to 1% or less of its initial value. From previous experiments[12] we know that the circulating quantity of that portion of the globulin containing the antibody is reduced only to an equal or lesser extent than the total protein, *i.e.* 50% or less. Practically this means that the measurement of such quantities as serum protein concentration, total circulating serum protein or any one of its components may give little if any insight into the *rate* of production or turnover of the protein.

Summary. The present experiment investigated the rate of loss of the capacity to produce antibody in comparison with the rate of loss of protein from body compartments including carcass, liver and plasma. Young adult male albino rats were tested at intervals from 0 to 100 days after initiation of the low protein diet for agglutinin and hemolysin production. Blood volumes, hemoglobin, serum protein, liver and carcass protein were determined. It was found that all protein compartments and the rate of antibody production fell at approximately linear percentage rates per day, the antibody production falling much faster than the rest. Statistically significant depression of the antibody titers was not reached till the 30th day with hemolysins and the 17th day with the agglutinins.

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[12] Benditt, E. P., unpublished observations.

16888

Chemotherapy of *Trichomonas foetus* Infections in Rabbits.*

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No satisfactory therapeutic agent has been established for the successful treatment of bovine trichomoniasis. *In vivo* studies in laboratory animals of the trichomonacidal properties of various chemicals may offer useful information for future treatment trials in cattle. The purpose of this paper is to present the results of a study on the effect of 58 compounds on *Trichomonas foetus* in the vagina of rabbits. The majority of the compounds were selected from 350 chemicals previously tested *in vitro* by Morgan and Campbell.[1]

Witte[2] apparently was the first to successfully infect rabbits with *T. foetus* by vaginal

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† Wellcome Research Fellow of the Animal Health Trust (from the Ministry of Agriculture and Fisheries Laboratories, Weybridge, England).

[1] Morgan, B. B., and Campbell, H. M., *Am. J. Vet. Res.*, 1946, 7, 45.

[2] Witte, J., *Arch. wiss. prakt. Tierheilk.*, 1933, 66, 333.

inoculations. Trichomonads were recovered from the inoculated rabbits intermittently for 26 days. Nelson[3] was successful in infecting 2 rabbits intravaginally. According to Nelson[4] and Byrne and Nelson[5] 38 rabbits which were given intravaginal injections of *T. foetus*, 32 or 84% developed self-limiting infections with an average duration of 23 days. MacDonald *et al.*[6] inoculated 76 rabbits intravaginally with *T. foetus* with an incidence of 67%. Wittfogel,[7] Stableforth and Scorgie[8] and Trussell and McNutt[9] were unable to infect rabbits by the intravaginal inoculation of *T. foetus*.

Nelson[4] was the first to use the rabbit as a test animal for various therapeutic agents against *T. foetus*. Attempts to rid the organisms from the vagina of infected rabbits with 3 pentavalent and 1 trivalent arsenicals and sodium bismuthyl tartrate were unsuccessful.

Experimental procedure. Virgin female rabbits, of different breeds, obtained from various sources were used in the experiments. The animals were 6 to 8 months of age and weighed from 5 to 8 lb.

The bacteria-free strain of *T. foetus* used in this work was isolated by Morgan and Wisnicky.[10] Forty-eight to 96-hour-old cultures were used. The organisms were cultivated on a modification of Schneider's citrate; whole egg and defibrinated bovine blood slants overlaid with buffered saline citrate solution with 5% bovine serum. The number of trichomonads was determined by hemacytometer counts. A standard suspension of 3 million organisms per ml was used for inoculation purposes.

Rabbits were inoculated with 10 ml of a trichomonad suspension into the vagina by means of a sterile glass tube 7 inches long and ¼ inch in diameter. In the initial screening tests only 1 rabbit was used for each drug. A rubber adapter was attached to the free end of the glass tube and the trichomonads introduced by means of a glass syringe.

Microscopic identification of *T. foetus* was made from recently collected vaginal samples. A sterile wooden applicator stick with a cotton pledget attached was enclosed in a glass tube and inserted into the vagina. The glass tube was withdrawn, the swab rotated and a mucus sample obtained. Material adhering to the cotton was mixed with 0.7% saline; placed on a slide and examined.

Throughout the course of these studies the external genitalia of the rabbits were washed with a disinfectant prior to inoculation of infective material or collection of mucus samples. All rabbits were sampled prior to the experiments and the material plated on blood agar to determine the presence of bacteria. In a number of rabbits a gram negative rod was isolated in pure culture. Whether this bacterium was part of the normal flora of the vaginal tract of the rabbit could not be determined; however, its presence apparently had no effect on the course of the subsequent *T. foetus* infection.

Vaginal inoculation of *T. foetus* infected from 20 to 80% of the rabbits. The factors responsible for variation in susceptibility are not well understood. Microscopical examinations were made during the first 5 days following the infective inoculation and only those rabbits which showed a considerable number of organisms in the collected discharges were utilized in the experiments. Where the infection appeared to be mild during this time the rabbits were discarded. By making this selection the infection persisted in the 30 untreated controls for a minimum of 20 days.

Fifty-eight rabbits were treated with various compounds after showing an infection for 5 days. Three drugs which inhibited *T. foetus* were further tested utilizing 10 rabbits for each drug and 10 controls. Liquids and jellies were introduced in the vagina with a glass

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[3] Nelson, P., *Arch. Path.*, 1937, **23**, 744.

[4] Nelson, P., unpublished thesis, Library, University of Wis., 1938.

[5] Byrne, H., and Nelson, P., *Arch. Path.*, 1939, **28**, 761.

[6] MacDonald, E. M., *et al.*, *J. Immunol.*, 1948, **59**, 295.

[7] Wittfogel, H., *Inaug. Diss. Hannover*, 1935, p. 68.

[8] Stableforth, A., and Scorgie, N., *Vet. Rec.*, 1937, **49**, 253.

[9] Trussell, R., and McNutt, S., *J. Infect. Dis.*, 1941, **49**, 453.

[10] Morgan, B. B., and Wisnicky, W., *J. Am. Vet. Med. Assn.*, 1942, **100**, 471.

TABLE I.

Therapeutic Agents Used for Treatment of Vaginal Infections in Rabbits Induced by *Trichomonas foetus.*

1. Phemerol (*p-tert*-Octylphenoxyethy-oxyethyldi-methyl benzyl-ammonium-chloride) (Liquid) (.01%)
2. Sulfaguanidine (Powder)
3. Povage (Silver chloride-thiourea complex salt + 3-chloro-4-hydroxy diphenyl + osmo-kaolin (Powder)
4. Vioform (5-chloro-7-iodo-8-hydroxyquinoline) (Powder)
5. Sodium bicarbonate (Liquid) (5%)
6. Carbarsone (p-Carbaminophenyl arsonic acid) (Powder)
7. Negatan (Polymerized disulphonic-dioxydimethyl-diphenylmethane acids) (Suppository 10% negatol)
8. Tyrothricin (Water soluble jelly) (.01%)
9. Ceepryn vaginal powder borated (Cetylpyridinium chloride (.5%) + boric acid + kaolin + dextrose) (Powder)
10. Entozon granulate (2,3-Dimethoxy-6-nitro-9-(*y*-diethylamino-*B*-hydroxypropylamino) acridine dihydrochloride + 2-orthoxy-6,9-diamino-acidrine lactate + amyl saccharine + sodium biborate) (Liquid) (2%)
11. Iodine (Tincture) (Liquid) (0.5%)
12. Lugol's Solution (Iodine (5 gr.) + potassium iodide (10 gr.) + water (100 cc)) (Liquid)
13. Trypaflavine (2,8-Diamino-10-methylacridinium chloride) (Liquid) (1%)
14. Proflavine dihydrochloride (3,6-diaminoacridine dihydrochloride) (Liquid) (3%)
15. Meta Cine Douche Powder (Citric acid + papain + lactose + methyl-salicylate + eucalyptol + menthol + chlorthymol) (Liquid) (3%)
16. Silver picrate (1% dispersed in kaolin) (Powder)
17. Zonite (Sodium hypochlorite + sodium hydroxide + sodium chloride) (Liquid) (3%)
18. Merpectogel (Phenylmercuric nitrate (1:24,000) + pectin jelly) (Jelly)
19. Caprokol (Hexylresorcinol 1:1000 + alcohol + water soluble jelly) (Jelly)
20. Chinosol (8-Hydroxyquinoline sulfate) (Liquid) (5%)
21. Amertan (5% tannic acid + merthiolate) (Jelly)
22. Gentian violet jelly-merthiolate (Jelly)
23. Lactic acid (Liquid) (5%)
24. Acetic acid (Liquid) (5%)
25. Penicillin 10,000 units per cc (total 100,000 units) (Liquid)
26. Streptomycin (1000 units per cc) (total 10,000 units) (Liquid)
27. Mercuric chloride (Liquid) (.01%)
28. Metaphen (4-nitro-anhydro-hydroxy-mercury-orthocresol (Metaphen 1:2500)) (Liquid) (.5%)
29. Copper sulfate (Liquid) (10%)
30. Floraquin (5,7-diiodo-8-hydroxy-quinoline (Diodoquin) + boric acid + lactose + anhydrose dextrose) (Powder)
31. Lysol (Cresylic acid + neutral soap + glycerine) (Liquid) (0.25%)
32. Sodium perborate (Liquid) (5%)
33. Phenylmercuric acetate (Liquid) (.01%)
34. Chiniofon (7-iodo-8-hydroxyquinoline-5-sulfonic acid) (Powder)
35. Picric acid (Liquid) (1%)
36. Pine Oil Disinfectant (Liquid) (5%)
37. Potassium permanganate (Liquid) (1%)
38. Hydroxyquinoline benzoate (Liquid) (5%)
39. Sulfo-merthiolate (Sodium p-ethylmercuri-thiophenylsulfonate) (Powder)
40. Malachite Green (Liquid) (1%)
41. Actidione (Liquid) (1%)
42. Subtilin (Liquid) (5%)
43. Bovoflavin-salbe German proprietary compound (Ointment)
44. Propylene glycol dipropionate (Suspension in water) (20%)
45. Sodium 3-(gamma-hydroxymercuri-*B*-methoxypropyl-4-hydroxybenzoate) (Liquid) (1%)
46. Negatan No. 34 (Solution) meta-cresol sulfonic acid + formaldehyde (Liquid)
47. Sulfo-merthiolate (sodium p-ethylmercuri-thiophenylsulfonate, Lilly) 1:1000 (Jelly)
48. Gentian violet 1% with merthiolate (sodium ethylmercuri thiosalicylate, Lilly) 1:1000 (Jelly)
49. Quinacrine hydrochloride (Powder)
50. Pantaquine (6-Methoxy-8-(5-isopropyl-amino-pentylamino) quinoline phosphate (Liquid) (10%)
51. Sodium recinoleate (Liquid) (5%)
52. Potassium dithioformate (Liquid) (5%)
53. Sodium isopropylsulfonate (Liquid) (5%)
54. Sodium hydrogen sulfasalicylate (Liquid) (5%)
55. Sodium diethyldithiocarbamate (Liquid) (5%)
56. 2 methoxy 6-chloro-9 (2'-diethyl-amino-4'-butylamino) acridine 2 HCl (5%)
57. 2-methoxy 6 chloro-9 (a-di-n-propylamino) acridine 2 HCl (Liquid) (5%)
58. 7-chloro-4-bis (dimethylamino-2-propylamino) quinoline (Liquid) (1%)

syringe (10 ml per animal). Powders were insufflated into the vagina with a small insufflator at the rate of ½ g per rabbit. After treatment the rabbits were examined at various intervals for 3 to 5 weeks and sacrificed after 6 to 8 weeks. The evaluation was based on one application of a given drug.

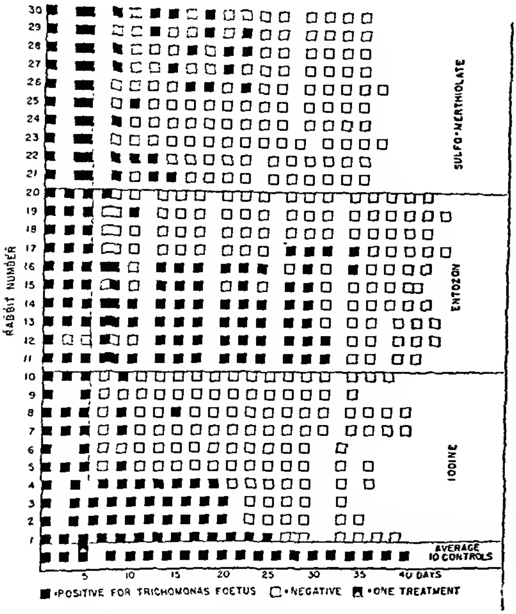

FIG. 1.

Results of *in vivo* tests with sulfo-merthiolate, entozon and iodine on *Trichomonas foetus;* 10 control rabbits.

Results. Table I indicates the 58 therapeutic agents used for treatment of the vaginal infections produced by *T. foetus;* of these compounds tested, only 3 appeared to reduce the duration of infection in the rabbits; entozon, iodine and sulfomerthiolate. Entozon, iodine and sulfo-merthiolate eliminated the infection, in 1, 4 and 5 days, respectively. The infection in all the other drugs tested persisted for at least 15 days following the treatment. None of the compounds tested at the dosage employed produced any symptoms of toxicity nor was there any apparent damage to the mucous membranes of the vagina on post-mortem examination. The 3 drugs when used on 10 rabbits each appeared to shorten the duration of infection when compared with 10 untreated controls (Fig. 1). The results, however, are considered inconclusive.

Summary. A standardized, controlled *in vivo* technic utilizing female rabbits has been described for testing the trichomonacidal properties of various therapeutic compounds against *Trichomonas foetus.* A total of 58 compounds was tested by this method. Only 3 compounds: entozon, iodine and sulfomerthiolate appeared to reduce the duration of infection.

16889

Effect of Alpha Naphthylthiourea (ANTU) on Serum Cholesterol in Thyroidectomized Dogs.

WALTER FLEISCHMANN, JANE L. STUBBS, AND WILLIAM P. MCSHANE.

From the Physiology Section, Medical Division, Army Chemical Center, Maryland.

Chronic poisoning of dogs with alpha naphthylthiourea (ANTU) is accompanied by a marked increase of the cholesterol content of the plasma. After discontinuing administration of ANTU the plasma cholesterol diminishes rapidly.[1] ANTU like other thiourea derivatives depresses the function of the thyroid gland. This underfunction leads to compensatory enlargement of the gland. Richter[2] who introduced ANTU as a rodenticide described hyperplasia of the thyroid in rats poisoned with the drug. The antithyroid effect of the drug has also been demonstrated by cytological studies of the thyroid of rats

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[1] Chanutin, A., Gjessing, E. C., and Ludewig, S., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 174.

[2] Richter, C. P., *J. Am. Med. Assn.*, 1945, 129, 927.

TABLE I.
Total Serum Cholesterol in Mg Per Cent.

Thyroidectomy	Treatment	Minimum	Maximum	Mean	S.E.*	Ratio free: total cholesterol
Before	None	145	210	187	7	.27
After	Thyroid	74	225	165	10	.30
,,	Thyroid + ANTU	160	458	288	13	.33
,,	Thyroid†	150	294	221	25	.33
,,	Thyroid‡	177	210	196	6	.35

* Standard error of the mean.
† One week after discontinuing ANTU.
‡ Two weeks after discontinuing ANTU.

treated with ANTU.[3] Hypothyroidism is known to be accompanied by a rise in plasma cholesterol. The increase in plasma cholesterol in dogs chronically poisoned with ANTU could therefore possibly be due to the "chemical thyroidectomy", caused by ANTU.[1]

In order to determine the possible role of the thyroid, it was necessary to study the effect of ANTU poisoning on the serum cholesterol of thyroidectomized dogs. The thyroid deficiency due to removal of the thyroid had to be compensated by administration of desiccated thyroid by mouth. From studies in hypothyroid children[4] and thyroidectomized rabbits[5] it was known that the elevated serum cholesterol due to thyroid deficiency decreases rapidly to normal values under treatment with the thyroid hormone.

Material and methods. Six adult female dogs weighing around 14 kg were used. After a pre-operative control period the thyroid and parathyroid glands were removed surgically and the parathyroid glands reimplanted. Calcium lactate was given intravenously and by mouth during the first few days after operation. Feeding with Desiccated Thyroid U.S.P. 640 mg daily was started a week after operation. As soon as the dogs were stabilized on a normal cholesterol level feeding with ANTU (10 mg per kg bodyweight daily) was started. This was continued for a period of from 3 to 10 weeks. After this period ANTU was discontinued but treatment with desiccated thyroid was continued. In one of these dogs the chronic ANTU poisoning was repeated after the cholesterol level had reverted to normal for about 2 months. All dogs were sacrificed after completion of the study. No thyroid tissue or only minute amounts were found at autopsy.

Free and total cholesterol were determined by a modified Schoenheimer-Sperry procedure.[6] A number of determinations of total cholesterol were made without digitonine precipitation on samples extracted with alcohol-ether. The results for total cholesterol obtained by this method were in good agreement with those obtained by the modified Schoenheimer-Sperry procedure.

Results. A summary of our experiments is given in Table I. Both from this summary and from the individual data on each experiment it can be seen that the thyroidectomized dogs treated with adequate doses of thyroid show normal serum cholesterol levels. The cholesterol levels of the operated dogs treated with thyroid tend to be slightly lower than during the pre-operative period. This is in agreement with the known fact that serum cholesterol levels in the adequately treated hypothyroid patient are in the normal or low normal range. Chronic poisoning with ANTU superimposed on the treatment with thyroid hormone results in a marked rise in serum cholesterol in every experiment. After discontinuing ANTU without changing the dose syringe (10 ml per animal). Powders were insufflated into the vagina with a small insufflator at the rate of ½ g per rabbit. After treatment the rabbits were examined at various intervals for 3 to 5 weeks and sacrificed after 6 to 8 weeks. The evaluation was based on one application of a given drug.

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[3] Jones, R. P., *J. Path. and Bact.*, 1946, **58**, 483.

[4] Wilkins, L., and Fleischmann, W., *J. Am. Med. Assn.*, 1941, **116**, 2459.

[5] Fleischmann, W., Shumacker, H. B., Jr., and Wilkins, L., *Am. J. Physiol.*, 1940, **131**, 317.

[6] Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th Ed. Blakiston Co., Philadelphia, 1945, p. 531.

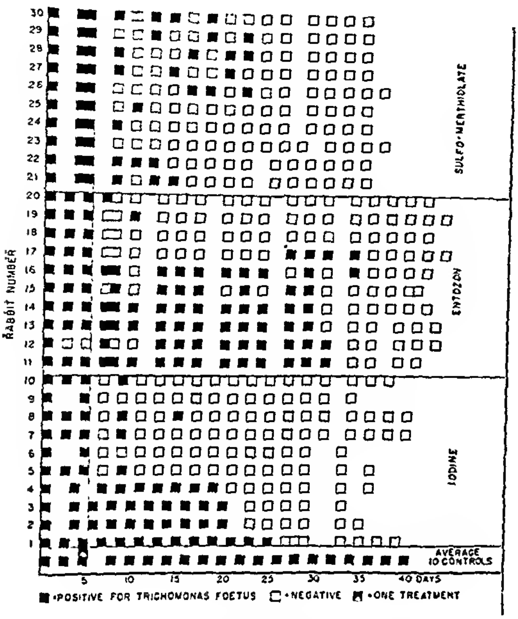

Fig. 1.

Results of *in vivo* tests with sulfo-merthiolate, entozon and iodine on *Trichomonas foetus;* 10 control rabbits.

Results. Table I indicates the 58 therapeutic agents used for treatment of the vaginal infections produced by *T. foetus;* of these compounds tested, only 3 appeared to reduce the duration of infection in the rabbits; entozon, iodine and sulfomerthiolate. Entozon, iodine and sulfo-merthiolate eliminated the infection, in 1, 4 and 5 days, respectively. The infection in all the other drugs tested persisted for at least 15 days following the treatment. None of the compounds tested at the dosage employed produced any symptoms of toxicity nor was there any apparent damage to the mucous membranes of the vagina on postmortem examination. The 3 drugs when used on 10 rabbits each appeared to shorten the duration of infection when compared with 10 untreated controls (Fig. 1). The results, however, are considered inconclusive.

Summary. A standardized, controlled *in vivo* technic utilizing female rabbits has been described for testing the trichomonacidal properties of various therapeutic compounds against *Trichomonas foetus.* A total of 58 compounds was tested by this method. Only 3 compounds: entozon, iodine and sulfomerthiolate appeared to reduce the duration of infection.

16889

Effect of Alpha Naphthylthiourea (ANTU) on Serum Cholesterol in Thyroidectomized Dogs.

Walter Fleischmann, Jane L. Stubbs, and William P. McShane.

From the Physiology Section, Medical Division, Army Chemical Center, Maryland.

Chronic poisoning of dogs with alpha naphthylthiourea (ANTU) is accompanied by a marked increase of the cholesterol content of the plasma. After discontinuing administration of ANTU the plasma cholesterol diminishes rapidly.[1] ANTU like other thiourea derivatives depresses the function of the thyroid gland. This underfunction leads to compensatory enlargement of the gland. Richter[2] who introduced ANTU as a rodenticide described hyperplasia of the thyroid in rats poisoned with the drug. The antithyroid effect of the drug has also been demonstrated by cytological studies of the thyroid of rats venous use of the plasma or whole blood. (1) It must not alter the plasma proteins in such a way as to make them toxic or antigenic. (2) The agent used must be nontoxic or must readily be converted to a nontoxic substance on standing or on suitable neutralization with a second nontoxic substance; (3) It should cause minimal or no changes in the immunological components of the blood; (4) It should not cause hemolysis nor increased fragility of red blood cells. In the present investigation, a member of the nitrogen mustard group, methyl-bis (beta-chloroethyl) amine hydrochloride has been chosen for study for a number of reasons. (a) This compound has been extensively studied as a chemotherapeutic agent against the leukemias and is available in purified form;[3] (b) Its cytotoxic action is believed to be due to its effect on nucleoproteins toward which it has been shown to react competitively to a marked degree;[4-7] (c) In buffered aqueous solution the compound readily hydrolyzes to form relatively nontoxic endproducts; (d) The mechanism of action of this substance on biological systems closely parallels that of ionizing radiations.

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[1] Chanutin, A., Gjessing, E. C., and Ludewig, S., Proc. Soc. Exp. Biol. and Med., 1947, 64, 174.

[2] Richter, C. P., *J. Am. Med. Assn.*, 1945, 129, 927.

Experimental. I. Virucidal and Bactericidal Action of HN2. In the virus experiments a New Jersey strain of vesicular stomatitis virus was used.[‡] The mice used were 3- to 4-week-old Swiss strain. After several mouse passages in our laboratory, the LD_{50} of the virus was found to be 0.03 ml intracerebrally of a $10^{6.8}$ dilution. A 10% mouse brain suspension was prepared in rabbit serum, distributed to ampules, and frozen and stored in liquid oxygen for subsequent experiments. The inoculum was always 0.03 ml intracerebrally in mice and the titer of

TABLE I. Virucidal Action of HN2 in Human Serum, Plasma, and Whole Blood.

Description % virus suspension	Time interval, days	LD/50 control	(D) Dosage mg/l vs. Mortality (M)								Final pH
			D	M	D	M	D	M	D	M	
1. 1% in 95% serum		$10^{5.2}$	500	3/8*							8.4
same plus 2% 0.15 M $NaHCO_3$	5		500	3/8							8.5
same plus 5% 0.15 M $NaHCO_3$	5		500	7/8							8.6
2. 1% in 95% serum	5	$10^{5.2}$	200	6/8	200 200†	8/8	400	6/8	500	3/8	7.9 to 8.4
			600	0/8	800	0/8	1000	0/8			
3. 5% in 95% serum	2	—	400	5/5	500	5/5	600	3/5	800	3/5	
	5	—	400	5/5	500	5/5	600	3/5	800	3/5	8.0 to 8.2
4. 2% in 100% serum	3	$10^{5.7}$	500	8/20	550	1/20	600	0/20	700	0/20	7.7 to 8.2
5. 1% in 98% citrated plasma‡	3	$10^{6.8}$	400	0/10	500	0/10	550	0/10	600	0/10	7.0 to 7.03
6. 1% in 98% citrated plasma‡	3	$10^{6.5}$	250	1/4	300	0/5	350	0/4	400	0/5	6.6 to 7.05
7. 1% in 90% citrated blood‡	3	$10^{5.8}$	400	1/5§	500	0/5	550	0/5	600	0/5	6.67 to 6.73
8. 1% in 90% citrated blood‡	3	$10^{-6.6}$	250	3/5	300	2/5	350	0/4	400	0/4	6.68 to 6.85

* Denominator denotes number of mice tested, numerator denotes number of deaths.

† Second 200 mg/l added after 24 hr.

‡ In citrated blood and plasma experiments, enough $CaCl_2$ was added to remove the citrate, in this case 0.01 cc of 5 M $CaCl_2$ per cc of blood and plasma. Also enough heparin to prevent clotting once citrate was removed. The addition was made just before inoculation of animals.

§ Original bottle had to be diluted 1:10 because of excess of $CaCl_2$ which was toxic to the mice.

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[3] Gilman, A., and Philips, F. S., *Science*, 1946, **103**, 409.

[4] Tenbroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 271.

[5] Rose, H. M., and Gellhorn, A., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 83.

[6] Fruton, J. S., Stein, W. H., Stahman, M. A., and Golumbic, C., *J. Org. Chem.*, 1946, **11**, 571.

[7] Gjessing, E. C., and Chanutin, A., *Cancer Research*, 1946, **6**, 593.

[‡] Received from Dr. Carl E. Duffy.

of thyroid extract given by mouth the serum cholesterol level gradually reverts to normal values in every experiment. The ratio of free to esterified cholesterol stays within normal limits during the whole experiment and seems not to be appreciably changed by chronic ANTU poisoning.

Discussion. The experiments demonstrate that chronic poisoning with ANTU produces a reversible rise in serum cholesterol in thyroidectomized dogs treated with adequate doses of thyroid hormone. From our experiments it cannot be determined whether ANTU influences the metabolism of cholesterol or the distribution of cholesterol between blood and tissues. We can assume therefore, that ANTU produces a direct effect on serum cholesterol quite independent from an indirect effect due to the antithyroid properties of the drug.

The large dose of thyroid hormone used (640 mg daily) without inducing signs or symptoms of hyperthyroidism reflects the tolerance of dogs to large doses of thyroid hormone. The dog, in proportion to its body weight, has apparently a much greater capacity than does man to inactivate exogenous thyroid hormone. This inactivation seems to take place in the extrathyroid tissues. Therefore both normal and thyroidectomized dogs can tolerate large doses of thyroid hormone without damage.[7]

Summary. Chronic poisoning with alpha naphthylthiourea (ANTU) produces a reversible rise in serum cholesterol in thyroidectomized dogs maintained on a dose of thyroid hormone adequate to prevent thyroid deficiency. This indicates that the effect of ANTU on serum cholesterol is at least partly independent from its property as an antithyroid drug.

We wish to thank Mrs. Aurora Bradford for her assistance in the cholesterol determinations, and Miss Frieda Faiman for statistical analysis of the data.

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[7] Danowski, T. S., Man, E. B., and Winkler, A. W., *Endocrinology*, 1946, **38**, 230.

16890

On the Chemical Sterilization of Blood and Blood Plasma.*

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The present study was undertaken in a search for a suitable means of chemically sterilizing human plasma and whole blood without so altering the blood components as to make them unsuitable for intravenous injection. The high incidence of homologous serum jaundice (4.5 to 7.2%) resulting from the injection of pooled plasma has become a serious problem in the use of pooled lyophylized plasma. At the present time only those methods involving the irradiation of plasma with ultraviolet light,[1] x-ray, or high speed electrons[2] have offered promise of a solution to this problem. Of these methods, only electron bombardment has appeared to offer any promise in the sterilization of whole blood. All of these irradiation technics require the use of highly specialized equipment which precludes the possibility of treating plasma or blood except at specially equipped plants.

The specifications for a chemical sterilizing agent are dictated by the future intra-

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† The authors are indebted to Dr. Elizabeth M. Yagle for the immunological data in this report.

[1] Wolf, A. M., Mason, J., Fitzpatrick, J., Schwartz, O., and Levinson, O., *J.A.M.A.*, 1947, **136**, 476.

[2] Trump, J. G., personal communication.

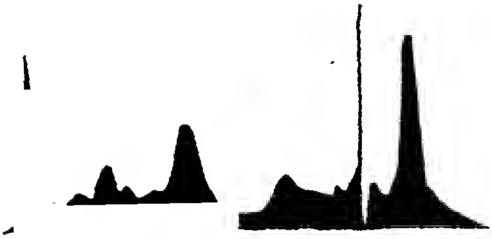

FIG. 1a.
Electrophoretic pattern of citrated plasma treated with 250 mg/l of HN2. (Barbital buffer, pH 8.6. r/2 0.01.)

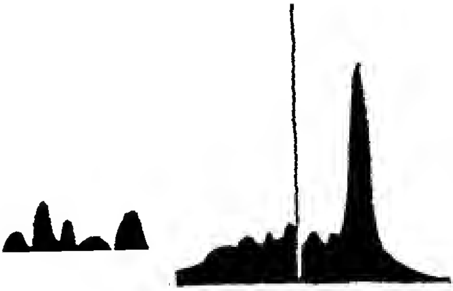

FIG. 1-b.
Electrophoretic pattern of control plasma from same sample.

ministered HN2 treated citrated whole blood as follows: 6 times with 100 ml of blood treated with 100 mg/1; once with 100 ml of blood containing 250 mg/1. Both animals remained in excellent condition throughout and following the treatment period. Their final weights following the last injection were 21 and 25¼ lbs respectively.

One normal individual has received a total of 5 doses of HN2 treated (600 mg/1) citrated type O plasma at approximately 2 week intervals as follows: 0.5 ml intradermally, 10 ml subcutaneously, 20 ml intravenously, 250 ml intravenously, and 250 ml intravenously. A second individual who has become locally sensitized to the unreacted beta-chloroethyl vesicant by repeated contact has received several intradermal, subcutaneous and intravenous injections of the same material without any evidence of sensitivity to the reaction products of the HN2 and plasma. No detectable reactions of any type have followed any of these tests. They clearly demonstrate the harmless nature of the final mixtures (10 days after mixing) and suggest that antigenic reactions are not likely to occur.

III. Biochemical Studies. Equally important with the virucidal activity and the nontoxic nature of the blood or plasma-HN2 reaction product are the alterations which occur in the plasma proteins and the red blood cells. A series of studies was, therefore, undertaken to ascertain the degree of alteration of some well known plasma proteins and of red blood cell properties and constituents.

A. Plasma Constituents: The extent to which added HN2 appears in the protein free filtrate has been roughly estimated by measurement of the amount of nitrogen in the protein free filtrate before and after the addition of various amounts of HN2 hydrochloride.

Most of the added HN2 appears in the protein free filtrate fifteen minutes after its addition while at 2 days and 5 days less than half of the added nitrogen is still present. The possibility of a part of the HN2 reacting with both NPN constituents and protein via the 2 available ethylenimonium groups should not be disregarded. Urea nitrogen determinations showed that no urea as measured by the urease method had disappeared.

The effect of HN2 on the A/G ratio has been determined. No measurable effect of 1000 mg/1 could be demonstrated 10 days later, while a marked difference was evident between samples stored at room temperature and those which were refrigerated. Additional evidence of the minor nature of the changes is seen in the electrophoretic pattern data prepared through the courtesy of Dr. Charles Janeway and Dr. John Newell, Massachusetts Blood Center, presented in Fig. 1. Visually the pattern exhibited a slight broadening of the albumin peak and increase of the ε-globulin peak.

The plasma fibrinogen concentration has

the control sample of virus was never less than 10^{-5}, even after standing for 5 days at 4°C. In each experiment the stock virus was added to the medium desired to make a concentration of 1 to 5% brain, the HN2 added in saline immediately after dissolving, and the mixtures and controls allowed to stand for 3 to 5 days in the icebox to allow the HN2 to react with the virus and the excess HN2 to hydrolyze. The material was then tested by inoculation in mice and any questionable deaths decided by passage of the mouse brain.

The results of studies on the virucidal action of HN2 in the presence of serum, ACD citrated plasma, and whole blood are presented in Table I. A consistent effect of pH has been observed, with more favorable results at pH values of 6.7 to 7.2 than at higher pH values. Destruction of the stomatitis virus is complete in ACD citrated plasma at 300 mg/l and in ACD citrated whole blood at 500 mg/l, while sterilization in serum required from 250 to more than 800 mg/l depending upon the pH and amount of serum added.

The bactericidal effects of HN2 have been studied on several organisms. In the absence of plasma, *B. coli* is killed by dilutions of HN2 as low as 100 mg/l. In inactivated citrated plasma at pH 7.1, the sterilizing dosage of HN2 is 450 mg/l after storage of the treated material 5 days in the icebox. Under the same conditions hemolytic streptococcus was killed at 450 mg/l, *Staphylococcus aureus* (strain 209 P) at 800 mg/l and *Pseudomonas aeruginosa* at 350 mg/l. Each organism was treated with HN2 at increments of 50 mg/l of HN2 up to 500, and increments of 100 mg/l up to 1200. 0.1 ml aliquots were used in each instance for subculturing and the treated tubes and controls were made up to contain initially about 1 million organisms per ml. A study of the time course of destruction of *B. coli* showed that the organisms were progressively destroyed over a 3 day period during which the active vesicant is present. At the end of this time the organisms will resume growth at 37° in the treated samples if not entirely killed.

II. Toxicity of HN2 treated Blood and Plasma. The toxicity of freshly dissolved HN2 has been extensively investigated and is of the order of about 3 mg/kg to various animals by the intravenous route. If allowed to stand in unbuffered solution there is an accumulation of the ethylenimonium ions which under certain circumstances may increase the toxicity beyond the original value due to the greater neurotoxic action of these intermediates.[9]

In bicarbonate buffer at pH 8.0 or in biological systems containing adequate buffering power to permit the degradation of the ethylenimonium ion at the end of 3 days at room temperature the chloroethyl and ethylenimonium compounds have disappeared and the toxicity decreased to the order of one thousandth its original value.[8] In the present experiments it has been found that appreciable toxicity may remain at 3 days in preparation stored in the refrigerator at 4-10°C, but after 5 days mixtures of blood, plasma or serum containing up to 1000 mg/l of HN2 are entirely innocuous to mice. Dosages as high as 5% of the body weight intravenously or 15% of the body weight intraperitoneally of citrated plasma containing 600 mg/l of HN2 hydrochloride are tolerated by mice without evidence of unfavorable reactions if the citrate effect is avoided with calcium chloride. The marked slowing of the decomposition by low pH values should, however, be emphasized.

Further evidence of the harmless nature of the end products of hydrolysis of HN2 in blood and plasma and of the probable lack of serious antigenic responses have been obtained in experiments on dogs and man. HN2 treated citrated plasma was administered to a dog weighing 18½ lb at 3 to 6 day periods as follows: 6 doses of 100 ml of plasma treated with 100 mg/l; twice with 100 ml of plasma containing 250 mg/l; once with 50 ml containing 2000 mg/l; twice with 100 ml containing 600 mg/l. Another dog initially weighed 23½ lbs and was intravenously ad-

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[8] Golumbic, C., Fruton, J. S., and Bergmann, M., *J. Org. Chem.*, 1946, **11**, 518.

[9] Golumbic, C., and Bergmann, M., *J. Org. Chem.*, 1946, **11**, 536.

TABLE IV.
Effect of HN2 on the Rate of Hemolysis of Red Blood Cells.

Dosage of NH2, mg/l	$NaHCO_3$	pH	Hemoglobin determined as oxyhemoglobin, g/100 ml of plasma	Hemoglobin determined as cyanmethemoglobin, g/100 ml of plasma
Control	No	6.70	1.28	1.30
450	"	6.63	1.46	1.50
600	"	6.69	1.40	1.38
800	"	6.67	1.39	1.40
1000	"	6.62	1.32	1.37
450	Yes	6.71	1.18	1.18
600	"	6.70	1.13	1.15
800	"	6.78	1.10	1.12
1000	"	6.73	1.14	1.17

(The ACD citrated blood was treated one hour after collection with the amount of HN2 indicated, with or without the calculated amount of $NaHCO_3$ to neutralize all 3 chlorides, stored without disturbing for 3 months, thoroughly mixed, centrifuged, and the hemoglobin determined in the supernatant plasma.)

ages of 450-600 mg per liter, careful studies have shown that the onset and rate of hemolysis of refrigerated whole blood is approximately equal in treated and untreated blood, if pH is adequately controlled. Observations on citrated human blood and rabbit blood have indicated that these are not affected by the sterilizing dosages of HN2. Dog blood, however, will not tolerate dosages of HN2 in excess of 250 mg/l. At 600 mg/l, the onset of hemolysis is rapid (within 24 hours) and hemolysis is almost complete within a week. This is in marked contrast to the behavior of human blood which remains in apparent good condition at the end of one week after treatment with as much as 1500 mg/l of HN2.

The results of application of various doses of HN2 upon red blood cell fragility has been investigated. Very little change was observed in fragility at sterilizing doses of HN2, but the cells became increasingly susceptible to hypotonic salt solutions as the dosage approached 1000 mg/l.

Discussion. It has been demonstrated that HN2 is capable of exerting a bactericidal and virucidal action in whole blood, blood plasma and blood serum without causing major alterations in the properties of either the plasma components or the red blood cells. These observations are supported by the findings of Tenbroeck and Herriott[3] and Rose and Gellhorn[1] on the virucidal action of this compound on 5 other viruses in the absence of blood. Because of the present *inability to transmit* to animals the agent of virus hepatitis, it has not been possible to study this virus until careful studies had been made upon the characteristics of treated plasma. It is felt that the present evidence is sufficiently strong to warrant the direct study of this virus as soon as a more extensive group of recipients have received the treated plasma.

The data presented in this paper strongly suggest that at least two variables are involved in the virucidal activity of HN2—the competition of the virus with plasma and whole blood components, and the effect of pH. Much of the effect of varying the percentage of plasma or serum on the virucidal activity of HN2 has now been traced to the buffering action of the blood. In contrast to the supposed advantage of a pH of about 8.0, it has been observed that virucidal action is greatly enhanced by decreasing the pH to 7.2 or below. It seems likely that the beneficial effect of a lower pH is due to a reduction in the rate at which HN2 decomposes and/or reacts with other competing substances.

On the basis of studies carried out to date, it seems likely that the required sterilizing dosage of HN2 will be no greater than 500 mg/l if the pH of the treated material is held at or below 7.2. ACD citrated blood pH values range from 6.7 to 7.0. Studies are now under way to establish the optimum pH for the virucidal and bactericidal action of HN2.

Summary. Methyl-bis (beta-chloroethyl) amine hydrochloride has been demonstrated to exert effective virucidal and bactericidal effects in the presence of either plasma, serum

TABLE II.
Effect of HN2 on Prothrombin in Acid Citrated Whole Blood at pH 6.7-6.8 Three Days After Treatment.

HN2, mg/l	Prothrombin, % fresh control	HN2, mg/l	Prothrombin, % fresh control
Control	82	650	17
Control with HCl eq. to 1000 mg/l	74	700	15
450	38	750	12
500	16	800	10
550	22	900	9
600	16	1000	6

TABLE III.
Effect of HN2 on Plasma Prothrombin of Citrated Plasma and Whole Blood Treated with 600 mg/l of HN2.

Hours	Prothrombin, % of normal control		
	Plasma	Whole blood*	Whole blood†
1	54	80	—
1½	60	66	66
3	46	46	50
20	42	46	46
27	37	46	46
44	37	42	46
51	37	40	40
68	34	44	48
77	34	52	52
92	34	44	52

* Plasma separated after one-half hour.
† Separated just before each prothrombin determination.

been measured before and after treatment with HN2. No decrease is produced by concentrations up to 1000 mg/l. Clot formation progressively fails with the addition of higher concentrations (complete inhibition at about 1%) even in the presence of large excesses of added thrombin.

Of all the substances studied to date, only prothrombin time (Quick's method) is markedly affected by the sterilizing dosage of HN2. Table II shows the effect of HN2 on apparent prothrombin activity and Table III shows the time course of the inactivation of prothrombin in plasma, whole blood, and whole blood separated one hour after addition of HN2. Whether the apparent inactivation of prothrombin is due to the effect of HN2 on prothrombin or accelerator globulin is being investigated.

Other substances studied to date included alkaline phosphatase, complement and immune bodies. None of these substances were markedly affected at 500 mg/l of HN2.

B. Effect of HN2 on Red Blood Cells. The possible use of chemical sterilizing agents in whole blood is predicated upon the ability of the chemical used to destroy the contaminating organisms without a concomitant destruction of the red blood cells or without so altering them as to render them short lived when introduced into the body. At the present time the *in vivo* survival of the red cells has not been adequately investigated but such studies have already been arranged.

Numerous samples of citrated whole blood have been treated with HN2 during the various studies carried out to date. Somewhat different results have been obtained on the 3 species studied. Human blood in ACD citrate-glucose solution have uniformly exhibited good storage qualities over periods up to 3 months as compared with the control bloods. In no case with dosages of HN2 hydrochloride of up to 800 mg/l have the treated specimens shown any evidence of deterioration greater than that of the same blood control. We have, in fact, sometimes observed that the treated bloods failed to exhibit hemolysis until several days after the untreated controls showed definite hemolysis. At doshe rate of 0.05 cc every 10 seconds.

reactions of the mouse to the timed us infusion of metrazol follow a de tern. Three "signs" of reaction follo n other in strict sequence as the concen ation of metrazol is increased in the blood stream. The first of these is the "first twitch," a sharp single twitching of the animal's entire body. This is followed very shortly by a "pseudoconvulsion," a series of clonic movements usually accompanied by an audible squeak. Very often the mouse pulls his head down under his body. The "pseudoconvulsion" is followed by alternate clonic movements and resting phases. The third sign, or "persistent convulsion," appears at the highest concentration of metrazol, and is the final end-point of the injection. The "persistent convulsion" consists of a tonic flexor component followed by a usually lethal tonic extensor component.

The doses of a 0.5% solution of metrazol at which the above-mentioned reactions occur were determined in control animals. Results appear for groups made up of mice weighing 15-17 g, and another series weighing 18-20 g (Table I). It will be noted that the thresholds of the mice weighing 15-17 g are lower, but that the mg/kg dosage is approximately the same.

In determining the efficacy of an anticonvulsant drug, observations were made on the ability of the compound to elevate the "first twitch," "pseudoconvulsion," and "persistent convulsion" thresholds. Also, the ability of a compound to protect the animals from death and to modify the severity of the clonic convulsions was considered.

The intravenous infusion of strychnine was likewise undertaken because of the chemical similarity of phenurone (phenyl acetyl urea) to myanesin (ortho-tolyl glycerol ether) and the fact that clinically a few patients report muscular weakness as a side effect of phenurone therapy.[4] Berger and Bradley[5] have shown that myanesin protects against strychnine convulsions. From our data this test

TABLE I. Intravenous Metrazol Infusion—Mice.

Groups	No. mice	1st twitch, cc	Pseudoconvul., cc	Persistent convulsion, cc	Type of seizure	Mortality, %	Time of death, min.
Normal, 15-17 g	40	0.14 ± .027 (1.00)	0.17 ± .037 (1.00)	0.45 ± .104 (1.00)	Tonic, flex.-ext.	100	Immed.
" 18-20 g	20	0.175 ± .026 (1.00)	0.23 ± .061 (1.00)	0.55 ± .181 (1.00)	" "	100	"
Tridione, 500 mg/kg 1 hr	20	0.37 ± .060 (2.64)*	0.44 ± .099 (2.57)	0.86 ± .003 (1.90)	" "	100	"
" 500 mg/kg 1½ hr	20	0.33 ± .058 (2.36)	0.40 ± .097 (2.37)	0.76 ± .109 (1.67)	" "	100	"
Phenurone, 400 mg/kg 1½ hr	20	0.32 ± .037 (2.29)	0.44 ± .067 (2.60)	0.83 ± .108 (1.83)	Clonic	30	82
Phenobarbital, 50 mg/kg 2 hr	20	0.36 ± .050 (2.55)	0.44 ± .062 (2.60)	0.85 ± .080 (1.89)	"	100	7.6
Myanesin, 300 mg/kg 10 min.	20	0.24 ± .043 (1.38)	0.32 ± .054 (1.39)	0.61 ± .088 (1.1)	"	100	27

* = Ratio: $\frac{\text{Threshold after treatment}}{\text{Normal threshold of corresponding wt group}}$.

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[4] Gibbs, F. A., personal communication.

[5] Berger, F. M., and Bradley, W., *Brit. J. Pharm. and Chem.*, 1946, 1, 269.

or whole blood. The required virucidal dosage of HN2 is below 500 mg/l of blood or plasma if the pH is at or near 7.0. The end products of decomposition of the added HN2 are essentially nontoxic if the pH of the system is not too low. No evidence of antigenic or other toxic reactions has been produced in two dogs and two humans receiving repeated injections of treated plasma. Human red blood cells withstand the application of virucidal dosages of HN2 without hemolysis and with only a slight increase in fragility. Complement, immune bodies, phosphatase and fibrinogen are only slightly affected by sterilizing dosages of HN2. Prothrombin time is markedly prolonged by HN2.

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Timed Intravenous Infusion of Metrazol and Strychnine for Testing Anticonvulsant Drugs.*

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The validity of the use of metrazol for the evaluation of anticonvulsant drugs has been well established.[1,2] When given to animals, subconvulsant doses of metrazol will elicit electro-encephalographic patterns similar to those found in patients with petit mal.[3] The marked antagonism between metrazol and 3,5,5-trimethyloxazolidine-2,4-dione (Tridione) and the success of this drug in the treatment of *petit mal* epilepsy further substantiate the validity of the metrazol antagonism test as a method for screening anti-epileptic compounds.

The present metrazol test method employs the subcutaneous route of administration whereby an effective dose of the anticonvulsant is injected into a group of animals, followed, after time is allowed for absorption, by a subcutaneous dose (usually 90 mg/kg) of metrazol known to be convulsant in the non-protected animal. If the animals fail to have seizures, a second group is given a higher dose of metrazol. This procedure is repeated until a dose is attained which produces typical convulsions. In this way the degree of protection (or metrazol antagonism) furnished by a drug in question may be ascertained.

For several years we have used a timed intravenous infusion of metrazol in mice, and have found it to have some distinct advantages over the previous procedure. A similar procedure has been evolved for the measurement of strychnine antagonism by anticonvulsant drugs.

Method. The apparatus employed consists of a cone-shaped mouse holder made of plexi-glass and attached to a metal base. The plexi-glass makes it possible to clearly observe the reactions of the animal within the holder. A slit, through which the tail of the mouse is pulled, extends the length of the cone at its top. Attached to the base is a small electric light connected in series through a transformer to a synchronous electrical timer. The synchronous timer is set so that the electric bulb lights every 10 seconds. The reflection of the light is transmitted throughout the transparent holder and thus is readily noted by the observer.

The mouse is pulled into the holder by drawing its tail through the slit in the cone, and the injection is made into a tail vein, using a 27 gauge needle. A 0.5% solution of metrazol or a 0.01% solution of strychnine sulfate is employed in all tests, and injected

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* This study was subsidized in part by grants from the Bristol Laboratories and the Mallinckrodt Chemical Works.

[1] Everett, G. M., and Richards, R. K., *J. Pharm. and Exp. Therap.*, 1944, **81**, 402.

[2] Goodman, L. S., Toman, J. E. P., and Swinyard, E. A., *Am. J. Med.*, 1946, **1**, 213.

[3] Goodman, L. S., Toman, J. E. P., and Swinyard, E. A., *Am. J. Med.*, 1946, **1**, 219.

TABLE III.
Intravenous Strychnine Infusion—Mice.

Groups	No. mice	Convulsion, cc	Mortality, %	Time of death
Normals, 15-17 g	60	0.19 ± .044 (1.00)	100	Immediate
” 18-20 g	20	0.21 ± .028 (1.00)	100	”
Tridione, 500 mg/kg 1 hr	40	0.28 ± .052 (1.48)	82.5	20 min.
Phenurone, 400 mg/kg 1½ hr	20	0.28 ± .042 (1.50)	60	28 min.
Phenobarbital, 50 mg/kg 2 hr	20	0.28 ± .033 (1.50)	65	19 min.
Myanesin, 300 mg/kg 10 min.	20	0.36 ± .052 (1.70)	30	42 min.

number of series, each at a different concentration of metrazol; thus, it calls for the use of many more animals and much more time, especially with effective drugs. For example: To test the efficacy of tridione (500 mg/kg) by the timed intravenous infusion method, one group of 20 animals was used. The total procedure (including weighing and injecting the animals) required 3½ hours. Employing the subcutaneous method to test tridione (500 mg/kg), and increasing the dose of metrazol in 25 mg/kg steps for each group of mice, 50 mice were used, 10 at each dosage level. The total procedure required 15 hours when each group was tested separately. Eighty mice were used to determine control responses, LD-50 and CD-50 for statistical analysis of the data for both groups.

The timed intravenous infusion method is as accurate as the subcutaneous method. Table II shows that the protection index for tridione (Ratio: tridione/control) is approximately the same by both methods of testing.

The sensitivity of the intravenous infusion method has been demonstrated frequently in the testing of new anticonvulsant chemicals. With one drug a 30% increase in dosage caused a 36% elevation of the protection index. In another instance a 20% increase in dosage caused the ratio to increase almost 200%.

The Timed Intravenous Infusion of Strychnine. The results obtained from tests made on phenobarbital, tridione, and phenurone are presented here to illustrate the validity of strychnine infusion as a standard laboratory procedure for screening anticonvulsant compounds (Table III). All 3 compounds are effective in elevating the strychnine threshold, phenurone and phenobarbital being slightly more antagonistic than tridione. 82.5% of the mice die in the tridione tests, while phenurone and phenobarbital show greater ability to protect the animals from death. Myanesin is the most effective of all since it affords protection from death in 70% of the animals and produces the greatest rise in thresholds.

Summary. The timed intravenous infusion metrazol test makes possible an accurate, economical, and rapid evaluation of anticonvulsant compounds. It presents distinct advantages over the subcutaneous method because: (1) fewer animals are needed in order to draw a statistically significant conclusion on the drug being evaluated; (2) less time is necessary to complete the testing procedure; and (3) it is equal in accuracy to the subcutaneous method.

TABLE II.
Intravenous and Subcutaneous Metrazol in Mice.

	Intravenous							
	Control group—80 mice			Tridione 500 mg/kg I.P. 1 hr—20 mice				
	Dose mg/kg	S.D. mg/kg	S.E. mg/kg	Dose mg/kg	S.D. mg/kg	S.E. mg/kg	No. of S.D. above control threshold	Ratio: Tridione/control
First twitch	42	7	.8	111	19	4	9.9	2.6
Pseudoconvul.	51	13	1.4	131	32	7	6.2	2.6
Persistent convul. and death	134	36	4	259	38	8.5	3.5	1.9
	Subcutaneous							
	80 mice			50 mice				
CD-50	55	8	1.7	124	37	6.7	8.6	2.3
CD-95	62			178				2.9
LD-50	99	17	2.7	169	25	5.5	4.1	1.7
LD-95	121			200				1.7

may further elucidate the site of action of potent anticonvulsants.

The apparatus and technique are essentially the same as the metrazol method. A 0.01% solution of strychnine is injected at the rate of 0.05 cc every 10 seconds in all tests, after time has been allowed for absorption of the compound in question. The endpoint is the tonic extension of the animal's hind legs, *i.e.*, the peak of the strychnine convulsion.

Strychnine thresholds were determined on two control groups of mice in which the animals weighed 15-17 g, and 18-20 g respectively (Table III).

Results. The results obtained with 3 anticonvulsant drugs (sodium phenobarbital, tridione, and phenurone) and myanesin illustrate the use of the timed intravenous infusion method. These drugs were injected intraperitoneally at dosage levels which produced slight central nervous system depression. The mice were tested at the peak of drug action (usually 30 to 120 minutes). Degree of threshold elevation (Table I) is expressed as a ratio of the mean of the thresholds of the protected animals to the mean of the thresholds of the appropriate group of control animals of similar weight.

Tridione (500 mg/kg) effectively elevates the metrazol threshold. However, it does not prevent death or modify the "persistent convulsions." Both phenobarbital (50 mg/kg) and phenurone (400 mg/kg) elevate the thresholds significantly. Phenobarbital modifies the "persistent convulsions" into clonic fits but fails to lower mortality. Phenurone modifies the "persistent convulsions" and prevents fatality in 70% of the animals. Myanesin raises the thresholds only slightly when metrazol is infused, but the lethal outcome is delayed.

For purposes of comparison, the results obtained from the application of the subcutaneous metrazol method using tridione at the same dose is presented (Table II). Control experiments were performed and it was found that 85 mg/kg of metrazol produced convulsions in 95% of the mice, severe seizures in 60%, and death in 20%. Metrazol, at a dose of 125 mg/kg subcutaneously, produced severe convulsions and death in all animals tested.

The greatest advantage of the timed intravenous infusion metrazol method is that it makes possible an accurate graded evaluation of anticonvulsant compounds requiring a minimum of time and effort. Since the concentration of metrazol within the animal's blood stream is increased at a constant rate during each *single* trial, a complete picture of the antagonistic properties of a drug may be obtained from *one* series of animals using increments in dosage of 12.5 mg/kg. The subcutaneous method, however, necessitates a

TABLE I.
Percentage of Survival of Infected 10-day Chick Embryos with Various Drugs.

No. of eggs	Therapy	Dose	% survival at 21 days*
96	Saline		0
12	Sulfathiazole	.1 mg	0
12	"	1 "	0
12	"	5 "	77
12	"	10 "	71
12	"	15 "	88
10	Sufladiazine	5 "	100
10	"	10 "	88
10	"	15 "	70
10	"	20 "	85
10	Sulfamerazine	5 "	66
10	"	10 "	75
10	"	20 "	77
10	Sulfapyridine	1 "	0
10	"	10 "	33
10	"	15 "	50
10	Acetarsone	.25 "	0
10	"	1 "	0
10	"	10 "	0
16	Penicillin	10 units	0
16	"	100 "	0
16	"	1,000 "	0
6	Streptomycin	10 "	0
16	"	100 "	0
16	"	1,000 "	0
16	"	10,000 "	0
20	Aureomycin	10 "	0
20	"	100 "	0
20	"	1,000 "	0
10	Sulfathiazole Sulfamerazine	6 mg of each	100
10	Sulfathiazole Sulfamerazine	12 mg of each	71

* Based on number of embryos living after subtracting inoculation deaths.

B. Congenital Toxoplasmosis in the Guinea Pig. Two gravid guinea pigs approximately one week from delivery were inoculated subcutaneously with 0.2 cc of 1:1,000 dilution of *toxoplasma*-containing peritoneal exudate. Five days later both guinea pigs delivered 2 living and one stillborn offspring. One of the stillborns appeared to have bilateral cataracts; sections made of most of their tissues for microscopic examination revealed no obvious toxoplasma. The living young were allowed to remain with their mothers until the mothers died about 10 days following the injection of toxoplasma. The offspring were sacrificed at varying periods up to 12 days of age in each instance toxoplasma were either found on stained imprints of body tissues or were recovered by means of inoculation into mice.

A third gravid guinea pig was infected one week before term in the same manner as described above, but before spontaneous labor ensued she was given ether anesthesia and 3 live young were delivered by Cesarian section to prevent the possibility of contamination via the birth canal. Toxoplasma were recovered from the mother's blood at delivery and were demonstrated in imprints of tissues in two of the offspring that died 24 hours later. Toxoplasma also were recovered from the brain of the third offspring that died at the age of 7 days from convulsions. However, we were unable to identify the organisms in sections of tissue from which they were isolated, even after a prolonged search.

C. Recovery of Toxoplasma from the Urine of Rabbits and Guinea Pigs. Two rabbits were inoculated intracutaneously with mixtures of toxoplasma and serum for the routine neutralization test. Two guinea pigs were inoculated subcutaneously with only a suspension of toxoplasma. After 8 days, un-

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Experimental Toxoplasmosis.

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Investigations by others have already shown that certain of the sulfonamides[1,2] have an inhibiting effect on the growth of toxoplasma in the mouse and rabbit, but that penicillin,[3] streptomycin,[4] and certain antiprotozoal[4,5] drugs have no apparent effect. Recently, it has been shown[6,7] that when toxoplasma are inoculated into the 10-day-old chick embryo, death of the embryo regularly occurs 5 to 7 days after inoculation. We have used chick embryos infected in such a manner to determine the therapeutic effectiveness of various drugs on the disease by comparing the survival time of control embryos with infected embryos which had been treated. Additional observations on the infected mouse, rabbit and guinea pig are included in this report.

Methods and Materials. The "R.H." strain of toxoplasma isolated by Sabin[8] from a fatal human case of encephalitis was used in these studies. It was maintained in this laboratory by continuous intraperitoneal or intracerebral passage in mice. Fertile hens' eggs incubated both before and after inoculation at 37°C were inoculated with 0.2 cc of a 10% suspension of either mouse brain or peritoneal exudate into the yolk sac, using a No. 22 needle 1 inch long on a tuberculin syringe. In all instances, the initial inoculation was made in the 10-day-old fertile egg and the drug to be tested was injected in a similar manner 24 hours later. Drugs used were prepared so that the amount to be evaluated was contained in 0.2 cc, the amount injected. The following controls were run with each drug tested: drug control, toxoplasma control, and saline control. All drugs used were from commercial lot preparations and were diluted with sterile saline solution to the proper dosage just before use. After inoculation each egg was candled daily until death or hatching occurred. Chicks that hatched were sacrificed at various ages and 10% suspensions of their livers and brains in physiological saline solution were inoculated into mice to determine the presence or absence of toxoplasma. In the mice, guinea pig and rabbit studies, depending upon the instance, the organisms were either given by mouth or injected subcutaneously, or intraperitoneally.

Results. A. Drug Studies. Sulfathiazol, sulfadiazine, sudfapyridine, sulfamerazine, and sulfathiazol-sulfamerazine (combined) in varying dosages prolonged the survival of the toxoplasma infected chick embryo as shown in Table. I. Penicillin, streptomycin, aureomycin,† and acetarsone had no effect on the survival of the infected chick embryo. In the infected embryos (treated with one of the sulfonamides) that survived and were allowed to hatch, toxoplasma were recovered from the livers by mouse inoculation, in every instance regardless of the age of the chick. In the sulfonamide treated embryos, dosages used below 5 mg per 0.2 cc afforded little or no protection against the toxoplasma; dosages above 20 mg per 0.2 cc appeared too toxic to the embryo to determine its effectiveness.

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* National Research Council Fellow in Pediatrics.

[1] Sabin, A. B., and Warren, J., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 19.

[2] Weinman, D., and Berne, R., *J.A.M.A.*, 1944, **124**, 6.

[3] Augustine, D. L., Weinman, D., and McAllister, J., *Science*, 1944, **99**, 19.

[4] Cross, J. B., and Anigstein, L., *Texas Rep. on Biol. and Med.*, 1948, **6**, 260.

[5] Warren, J., and Sabin, A. B., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 15.

[6] Warren, J., and Russ, S. B., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 85.

[7] MacFarlane, J. O., and Ruchman, I., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 1.

[8] Sabin, A. B., *J.A.M.A.*, 1941, **116**, 801.

† Aureomycin was kindly supplied by the Lederle Laboratories.

16893

Histamine in Rat Plasma; Correlation with Blood Pressure Changes Following X-irradiation.

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Although considerable has been written to indicate that there is a close correlation between the conditions of shock and the liberation of a histamine like substance from damaged tissue[1,2,3] it is only recently that the publications of Prosser, Painter and Moore[4] and others[5,6] suggest that a similar histamine shock syndrome can consistently be produced with mid-lethal dosages of X-rays. Their observations indicate that with such concentration of X-rays the toxic products of tissue breakdown reach their highest concentration at about 2 hours after irradiation.

In view of the fact that no definite statement is available as to whether the toxic product liberated is actually histamine or not, it will be the object of this investigation to demonstrate that the toxic product in the blood stream following irradiation is histamine, and that the level of assayable histamine in blood is highest about 2 hours after irradiation and again about 5 days later, at which times the blood pressure of the animals is at its lowest point.

Experimental. In the experiments to be reported, adult rats were used irrespective of sex. After irradiation, the rats were used either for the collection of blood samples (enough for 2 cc of plasma) to be assayed for histamine at various time intervals, or for blood pressure determinations over the corresponding time interval. In making these determinations a total of 21 rats were used for making histamine assays at various time intervals, varying from .5 to 24 hours after irradiation; 5 other rats were used to follow variations in blood pressure, determined at 15 minute intervals for 4 to 5 hours after the radiation was given. After once being irradiated, never was a rat used for a second experiment.

The irradiation techniques used were developed by Quastler and Kirschner.[3] The rats were placed in a cell cut out of presswood, large enough to accommodate 3 rats. The factors for the raying were: 200 KVp without a filter. The distance from the target electrode to the top of the rats' cell was 50 cm. One-half of the dose was administered from the top and one-half from the bottom. By raying in this fashion a fairly homogeneous distribution of dose is attained. The dose rate was 100 r per 2 minutes and 24 seconds, and the actual mid-lethal dose given each rat was 600 r.

When blood pressure measurements were taken on the intact rat to record the severity of the effects of irradiation, a slight modification of the method described by Byrom and Wilson[7] was used. It consisted essentially in using a plethysmograph especially designed to fit the rat's tail.

The method used for preparing extracts for histamine assay was that developed by McIntire, Roth and Shaw.[8] This method appears to be superior to others available[9,10,11]

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[1] Moon, V. H., Oxford University Press, 1938.

[2] Wiggers, C. J., *Physiol. Rev.*, 1942, **22**, 74.

[3] Kirschner, L. B., Univ. of Ill., 1947, (Master's Thesis).

[4] Prosser, C. L., Painter, E. E., and Moore, M. C., to be published in Atomic Energy Commission Technical Series.

[5] Painter, E. E., and Moore, M. C., *Fed. Proc.*, 1948, **7**, 90.

[6] Prosser, C. L., Painter, E. E., Lisco, H., Brues, A. M., Jacobson, L. O., and Swift, M. N., *Radiology*, 1947, **48**, 299.

[7] Byrom, F. B., and Wilson, C., *J. Physiol.*, 1938, **93**, 301.

[8] McIntire, F. C., Roth, L. W., and Shaw, J. L., *J. Biol. Chem.*, 1947, **170**, 537.

[9] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

[10] Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, 1935, **85**, 1.

[11] Schild, H. O., *J. Physiol.*, 1942, **101**, 115.

der ether anesthesia the abdominal walls were opened sterilely and the bladders were aspirated with needle and syringe. In each instance the urine was very concentrated and so was diluted 3-fold with saline solution and then inoculated intraperitoneally into mice. Approximately 7 days later all of the mice became ill with ascites and toxoplasma were found in large numbers.

On 3 occasions mice were likewise infected with toxoplasma, both intracerebrally and subcutaneously, but toxoplasma were not recovered from the urine which was removed sterilely and inoculated into other mice.

D. Failure to Infect Mice with Toxoplasma Given Orally. On 5 occasions 7 mice were fed water containing millions of fresh toxoplasma in suspension, most of which was consumed 24 hours after preparation. None of these mice developed toxoplasmosis during the one month period of observation. Mice were likewise infected with toxoplasma intracerebrally and intraperitoneally and placed in cages with healthy litter mates to ascertain if infection could be acquired by cannibalism. In all instances the infected mice died 4-5 days after inoculation and were consumed by the normal litter mates. Under such circumstances, none of these litter mates developed toxoplasmosis.

E. Failure to Recover Toxoplasma from the Infected-Immune Rabbit. Three rabbits that had been used for the rabbit neutralization test but had recovered from their infection and had a high titer of complement fixing antibodies in their sera were sacrificed. The rabbits were selected so that one had recovered from its infection 1 month, one but 3 months, and one a considerably longer time, namely 19 months. In each instance sections of the brain, liver, and spleen were removed sterilely from the rabbit and made into a 10% suspension with saline. These suspensions (0.5 cc) were inoculated intraperitoneally into each of 3 mice who failed to develop toxoplasmosis after one month of observation.

Discussion. The results of the chick embryo method for testing the effectiveness of various drugs on toxoplasmosis compares favorably with previous studies done in intact animals.[1-5] This technique is relatively simple and non-time-consuming. Although it was thought that acetarsone produced some clinical improvement in acute toxoplasmosis in certain patients,[9] tests in the chick embryo showed no effect of the drug on the organisms. Aureomycin is known to be effective against experimental brucella infection in the chick embryo,[11] however, we were unable to show any inhibiting effect of the drug on the growth of toxoplasma.

While congenital toxoplasmosis was produced in the guinea pig, as proven by isolation of the organism from the brain, examination of the tissue sections microscopically revealed no organisms. This confirms our impression of the difficulty in making a diagnosis from pathologic specimens without the aid of biologic tests.[9]

Since we were unable to infect mice orally with toxoplasma, transmission from animal to animal does not appear to occur via the gastrointestinal tract. The significance of the presence of toxoplasma in the urine of infected rabbits and guinea pigs is not clear at the present time. Apparently, the infected rabbit, as well as the monkey[10] in certain instances, can recover completely from the infection.

Summary. The effectiveness of certain of the sulfonamides and the ineffectiveness of certain antibiotics in suppressing toxoplasmosis in the chick embryo has been demonstrated. Congenital toxoplasmosis was produced in the guinea pig.

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[9] Adams, F. H., Adams, J. M., Kabler, P., and Cooney, M., *Pediatrics*, 1948, **1**, 511.

[10] Sabin, A. B., and Ruchman, I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 1.

[11] Magoffin, R. L., Shaffer, J. M., and Spink, W. W., personal communication.

level of histamine concentration in the blood and the lowest point of blood pressure fall (Fig. 1). As the graph indicates, following this fall in blood pressure there occurs a marked compensation within an hour that exceeds the normal pressure at the beginning of the experiment. Although there is no satisfactory explanation for this change in blood pressure after the marked fall, it should be mentioned that when this rise does occur there is no measurable histamine present in the blood stream.

In a limited number of experiments in which the rats were followed for a longer time after irradiation than those just described, it was interesting to find that following the first 4 or 5 hours period after mid-lethal dosage of X-rays, even though the histamine in the blood was not measurable, the animals' blood pressure remained relatively low compared with normal, and continued to fall slowly to levels of 60 to 70 mm of Hg by the 4th to 6th day after irradiation. There also occurred in all the animals studied a marked loss in appetite and weight during this same period.

From this critical point, however, the rats either became progressively more depressed and died at the end of about 10 days, or showed a marked recovery in appetite, weight, blood pressure, and general appearance and survived the 600 units of X-irradiation. It was also interesting to note that if histamine assays were run on rats in these experiments there occurred a gradual increase in assayable histamine starting the 2nd day after irradiation, until on the 5th day, the rats showed nearly 1.0 γ of histamine (by assay) per 2 cc of blood plasma extracted. If the rats survived this critical 4 to 6 day period and showed signs of recovery, there was no evidence of histamine present in the blood by the 9th or 10th day after irradiation.

From these experiments it would appear that the low blood pressure correlates with high levels of histamine in the blood plasma which is very suggestive of the theory that the cause of death after irradiation is of the nature of circulatory failure or a condition simulating shock, an observation that was earlier suggested by Prosser, Painter and Moore.[4] Although no explanation can be advanced concerning the mechanism responsible for the appearance of histamine after irradiation, it is believed that the cyclic appearance of histamine at a 2 hour and a 5 day period after irradiation offers suggestions for further investigation of the problem in question.

Summary. 1. Rats, after X-irradiation with mid-lethal dosage (600 r) show assayable histamine in concentrations of 1 to 2 γ per 2 cc of blood plasma at 2 hour and 5 day intervals following the irradiation.

2. Rats irradiated similarly to those used in the histamine assay experiments show a fall in blood pressure that correlates with the appearance of histamine in blood plasma and strongly suggests that histamine is responsible for the lowered blood pressure.

3. Rats, from 4 to 6 days after a mid-lethal dosage of irradiation show a loss in appetite and weight, and a critical fall in blood pressure from which only 50% will recover. The rest die within 9 or 10 days after the irradiation was given.

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Chorio-meningo-enecphalitis Following Inoculation of Newcastle Disease Virus in Rhesus Monkeys.*

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Newcastle Disease Virus (NDV), commonly the cause of a disease of fowl, patently, may, because of intimate association with man, cause human disease. Recognized syndromes in man caused by NDV include conjuncti-

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* Aided by a grant from the United States Public Health Service.

in that very small amounts of histamine can be detected and also that only histamine is said to be extracted from the blood sample being studied and not other impurities. The essential features of the method are that the histamine can be removed from the aqueous sample almost in its entirety with N-butanol, and that then the histamine can be eluted from the butanol by means of the cation exchange medium, cotton acid succinate. Following this the histamine is washed from the cotton acid succinate with a small volume of dilute hydrochloric acid, and the resulting solution is neutralized with weak sodium hydroxide to give an isotonic solution suitable for bioassay. The actual assay for histamine is carried out by recording blood pressure changes in the anesthetized cat (Nembutal).

TABLE I.
Amount of Histamine Present in 2 cc Samples of Blood Plasma After Irradiation (600r).

Rat No.	Sampling time after x-ray (hr)	Histamine present (γ)
A4	.5	.0
A13	.5	.0
B1	1.5	1.0
A2	2.	1.0
A3	2.	2.0
A5	2.	2.0
A15	2.	1.2
A20	2.	2.0
A22	2.	1.4
B2	2.	1.2
B3	2.	1.2
C1	2.	1.6
C2	2.	1.6
B4	2.5	.4
A21	3.	.0
A23	3.	.0
A24	4.	.0
C3	10.	.0
C4	16.	.0
A25	18.	.0
A26	24.	.0

Results. In reporting the results obtained, particular emphasis will be placed on the correlation between the appearance of assayable histamine in the blood plasma and the maximum fall in blood pressure occurring approximately 2 hours after the mid-lethal dose of radiation was given (600 r). The data obtained for the actual amount of histamine present in terms of gamma per 2 cc of blood plasma is presented in Table I. That the assayable compound in these experiments is histamine is supported by the fact that the method used for isolating the compound is specific for histamine. It was also found that if a known amount of assayable histamine in gamma concentration which was previously extracted from a blood sample of an irradiated rat, is added to a similar amount of histamine diphosphate, the fall in blood pressure in the test cat was consistently twice that which occurs when the histamine diphosphate is injected alone. It is believed that if the extractive is not actually histamine it should not produce so clear cut an additive effect as these experiments indicated.

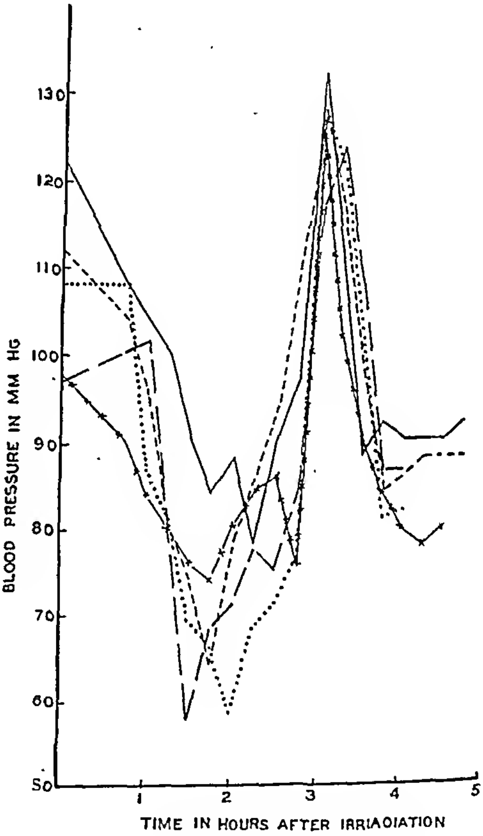
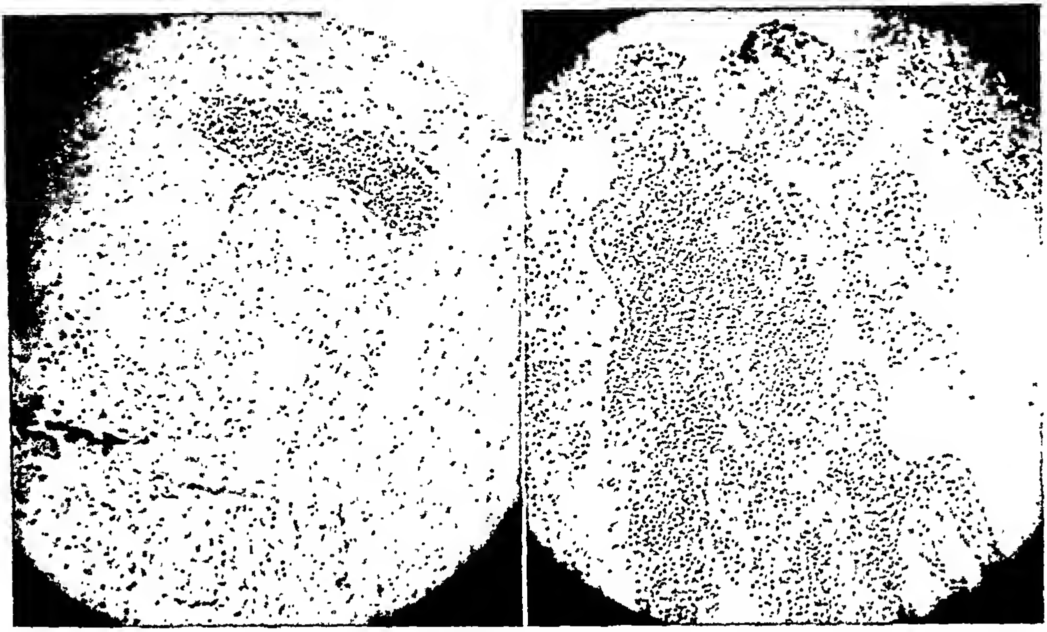


FIG. 1.
Effects of irradiation on blood pressure in the rat.

It was interesting to note that along with the appearance of histamine there occurred a marked fall in blood pressure which showed an excellent correlation between the highest



(1) (2)

FIG. 2.
Lesions in the (1) rhombencephalon and (2) choroid plexus of a rhesus monkey following intracerebral inoculation with Newcastle Disease virus.

mation of choroid plexus (Fig. 2). Perivascular cuffing, neuronecrosis and neuronophagia were best seen in the rhombencephalon, particularly in juxtaposition to the 4th ventricle. No changes were seen in the spinal cord of the monkeys inoculated with NDV infected allantoic fluid. In the passage monkey additional pathologic changes were found. These changes consisted of extensive inflammatory and degenerative changes in the gray matter. particularly in the anterior horns of the spinal cord.

Discussion. Heretofore, fowl and hamsters have been found susceptible to Newcastle Disease Virus. Now another host, the *rhesus* monkey has been found susceptible to NDV.[‡] The pathogenesis of NDV in rhesus monkeys requires additional clarification. At the height of illness NDV can be detected in CNS tissue and occasionally in the blood. but not in cerebrospinal fluid. If the pathological lesions only are considered it would appear that NDV may have been disseminated in cerebrospinal fluid. (CSF) The apparent absence of virus in CSF. in contrast to the presence of it in blood and CNS tissue may indicate widespread proliferation of virus. Further study is necessary in order to determine whether NDV resembles in pathogenesis experimental lymphocytic choriomeningitis or possibly poliomyelitis.

Summary. The "Manhattan" Strain of Newcastle Disease Virus produced in rhesus monkeys a chorio-meningo-encephalitis.

[‡] This virus causes encephalitis in cotton rats also.[4]

vitis,[1] and an influenza-like illness.[2] Recently, Howitt, Bishop, and Kissling[2] have pointed out that NDV may cause a mild CNS disease indistinguishable from abortive poliomyelitis in infants and children. In view of this last mentioned observation a study was made and Newcastle Disease Virus has been found to cause a CNS disease in rhesus monkeys. The results of this study are reported herein.

Materials and methods. Virus. The "Manhattan" strain of NDV[†] was grown in 10 day old embryonated eggs. A pool of infected allantoic fluid (12th passage) was made and stored at –70°C. The infected fluid had an embryo MLD in dilution 0.1 cc $\times\ 10^{-8.5}$. The hemagglutinin titer (fowl RBC) was 1:640. The hemagglutinin and hemagglutinin-inhibition tests were done with modifications as described by Florman.[3]

Monkeys. Rhesus (*Macaca mulatta*) monkeys were used. Each monkey received 0.8 cc of infected, undiluted allantoic fluid in the brain. Monkeys were observed twice daily. Temperature records were kept. Whole blood, serum and cerebrospinal fluid were obtained before and at intervals following inoculation. The CNS obtained from monkeys was examined histologically; fresh material was passaged to monkeys, rodents and embryonated eggs.

Results. Five monkeys were inoculated with infected allantoic fluid. All became sick. One died on the 6th day (following inoculation): one other was sacrificed on the 7th day. The remaining 3 monkeys recovered.

Clinical course. The clinical course of one monkey appears in Fig. 1. Following an incubation period of 2 to 5 days fever (104 to 105°F) occurred. With the onset of fever monkeys became agitated, and tremulous. They were grossly awkward in locomotion. The fever lasted 2 to 7 days. Convalescence was slow; symptoms lasted 7 to 10 days following abatement of fever. Cerebrospinal fluid obtained from 3 monkeys in the acute phase of illness was cloudy. The cell count ranged from 37 to $250/mm^3$. Mononuclear cells predominated.

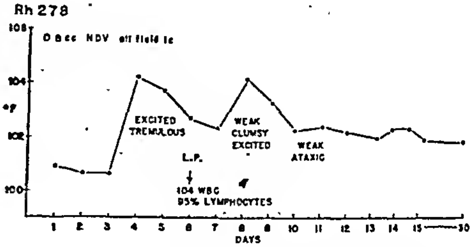


FIG. I.

Response of a rhesus monkey following intracerebral inoculation of Newcastle Disease virus.

Serologic Studies. Hemagglutinin-inhibition tests with pre-inoculation, acute and convalescent serums showed an 8 to 32 fold rise in NDV antibody level. Specific complement-fixation was obtained with NDV infected, allantoic fluid. No complement-fixation was obtained with lymphocytic choriomeningitis infected mouse brain.[6]

Passage. The CNS of a rhesus monkey sacrificed during the acute phase of illness was inoculated intracerebrally into 2 rhesus monkeys, Swiss mice, hamsters, guinea pigs, cotton rats and embryonated eggs. Chick embryos died; NDV was recovered from them. A monkey sickened. The sick monkey was unable to sit up on the 30th day. There was ipsilateral weakness of the right arm and leg. All other animals remained well (31 days).

Pathology. The CNS histology was characterized by (a) encephalitis, (b) focal meningitis, and (2) inconstant and intense inflam-

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[1] Burnet, F. M., *Med. J. Australia*, 1943, **2**, 313.

[2] Howitt, B. F., Bishop, L. K., and Kissling, R. E., *Am. J. Pub. Health*, 1948, **38**, 1263.

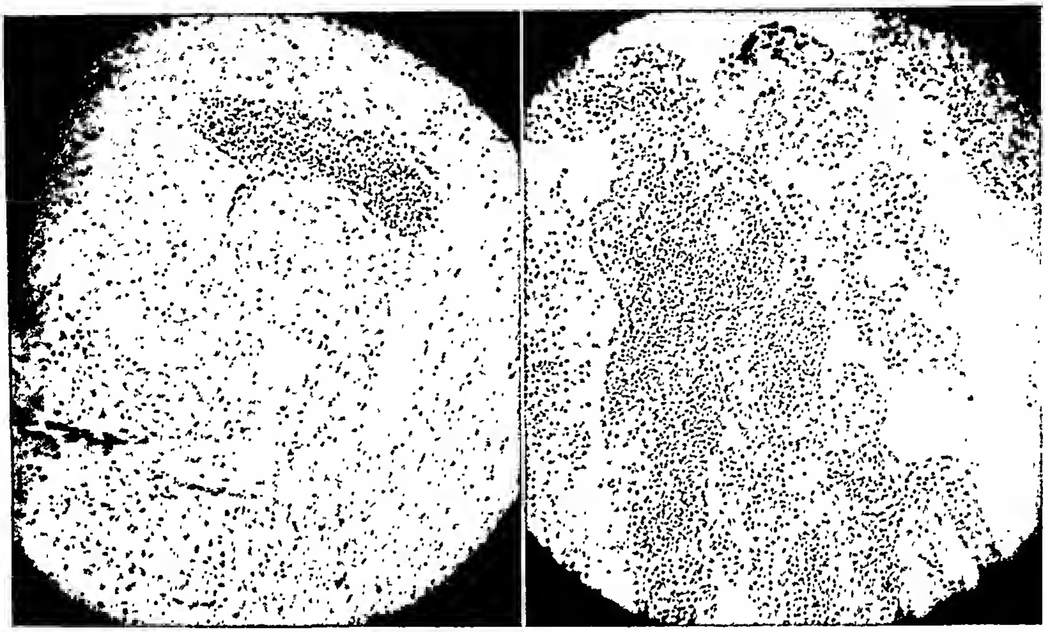
[†] We are indebted to Dr. L. D. Bushnell, Department of Bacteriology, Kansas State College, Manhattan, Kansas, for the "Manhattan" strain of Newcastle Disease Virus. On its receipt, the virus was in the 8th passage in the embryonated egg. Infected allantoic fluid (12th passage) inoculated intracerebrally in Swiss mice, guinea pigs, hamsters, and cotton rats, caused illness in cotton rats[4] and hamsters.[5]

[3] Florman, A. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 548.

[4] Wenner, H. A., unpublished observations.

[5] Reagan, R. L., Lillie, N. E., Hauser J. E., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 293.

[6] Espana, C., and Hammon, W. McD., *J. Immunol.*, 1948, **59**, 31.



(1) (2)

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Summary. The "Manhattan" Strain of Newcastle Disease Virus produced in rhesus monkeys a chorio-meningo-encephalitis.

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‡ This virus causes encephalitis in cotton rats also.[4]

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Effect of Subcutaneous Injury on Tumor Growth in the Mouse.*

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Several devices were explored for inducing inflammation under the skin of the mouse, to be followed by tumor implantation. Among these were the injection of acid, alkaline, or distilled water; the implantation of a small wad of glass wool; the injection of an air bleb, alcohol, turpentine, or histamine; and mechanical separation of the skin from the abdominal muscle wall. None but the last mentioned of these irritants produced consistent alterations in the growth of implanted tumor fragments. The type of inflammation induced by mechanical injury was, therefore, studied in detail in respect to its effect on tumor growth, and this effect comprises the main subject of this paper.

Procedure. A sharp-pointed 3-inch surgical scissors was inserted under the skin in the inguino-abdominal region and directed anteriad to a point about one-fourth inch from the axilla. With the position of the scissors' fulcrum lying at the point of original entry, the scissors was opened broadly so that the outer surfaces of its shears radically cleaved the skin from the underlying muscle tissue, without however puncturing the skin. Local bacterial infection was not evident in mice so treated, even when rigid surgical asepsis was not observed.

Groups of mice were so prepared for tumor implantation. Into some of these mice, fragments (approx. 2 mm^2) of sarcoma 180 were implanted immediately after the cleavage operation by inserting a trocar through the incision made by the scissors; the seeding fragment was deposited as close as possible to the center of the cleaved area. Fragments of comparable size and from the same donor tumor were implanted in the corresponding region of mice which had not been given the cleavage treatment; these served as controls.

Additional experiments were set up in which groups of cleavage-treated mice received tumor implants at various intervals after cleavage: at 24 hours after cleavage, at 48 hours, 72 hours, 6 days, and 12 days. In each instance tumors were implanted into parallel control groups of non-cleaved animals. The implanted fragment was allowed to grow for the usual 6 to 7 day period, at the end of which the tumorous growth was dissected out and wet-weighed. Before weights were taken, a puncture was made of the dissected tissue mass and exudative fluid was drained off. Rockland white male mice of 18-22 g were used throughout.

Results. For the first 4 days following fragment implantation into the cleaved subcutaneous area, tumor growth appeared to progress normally and was indistinguishable topographically from that of the controls. Between the 4th and 6th day after implantation a very marked swelling would become apparent in the area where the implant was deposited. Within 24 to 48 hours after the onset of this swelling, the experimental tumor appeared to attain a mass of from 5 to 10 times that of the control tumor (Plate I, Fig. 1 & 2). Upon dissection it was found that an extensive vesicle had developed throughout the cleavage area; and that a bleb of exudate had formed in close association with the growing tumor fragment (Plate I, Fig. 3). The vesicle fluid accounted for about half the total volume of the swelling. By the 9th day a hardening of the soft mass became evident. By the 12th day the edematous fluid was considerably replaced by the ingrowing tissue: the mouse in all such cases died before this process was complete, death usually occurring between the 12th and 15th day after implantation.

Animals implanted with tumor fragments immediately after cleavage exhibited the

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* The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council of the U. S. Public Health Service.

TABLE I.

Weights of tumors (sarcoma 180) dissected from mice during the 6-7 day period after implantation. Control mice had received no previous treatment; others were subjected to skin-muscle cleavage at various intervals before implantation (see text).

		Interval between cleavage operation and time of *tumor fragment implantation*			
Exp. No.	Control g	0-hr g	24-hr g	48-hr g	72-hr g
1	.15	.72	.47		.58
	.28	.39	.21		.42
	.15	.59	.47		.27
	.30	.22	.73		.12
	.20	.63	.12		.41
	.06				
	.25				
2	.20			1.00	
	.18			.45	
	.33			.54	
	.33			.41	
	.24			.67	
				.66	
				.16	
3	.25	.77	.48		.32
	.18	.89	.53		.47
	.18	.55	.63		.54
	.33	.47	.13		.24
	.20	.47	.86		.41
	.42	.19			.44
	.23				.20
	.18				
4	.46	.69	.46	.62	
	.51	.45	.31	.84	
	.26	.36	.33	.39	
	.29	.25	.81	.51	
	.27	.45	1.17	.61	
	.31	.71	.70	.70	
		.45	.18	.21	
		.87			
5	.39	1.10			
	.26	.86			
	.24	.31			
	.20	.96			
	.16	.28			
	.22	.74			
		.90			
Mean tumor wt	.25	.59	.50	.54	.36

above-described effect to an extent not distinguishable from that in animals in which the implantation was made 24 or 48 hours after cleavage. The effect showed signs of lessening in animals implanted 72 hours after cleavage; was barely apparent in those implanted 6 days after cleavage; and was completely absent in animals implanted 12 days after cleavage, at which time, apparently, complete healing of the original injury had occurred.

The data of Table I indicate that the mean weight of tumor tissue from cleaved animals sacrificed 6 to 7 days after implantation was in the order of twice that of the non-cleaved controls. It was noted, on the other hand, that had weighing been done at a later date, say during the 8-10 day period, the difference between cleaved and control groups would probably have been recorded as considerably greater. In Table I it will be noted, too, that the growth-enhancement effect occurred in

16895

Effect of Subcutaneous Injury on Tumor Growth in the Mouse.*

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From the Haskins Laboratories, New York City.

Several devices were explored for inducing inflammation under the skin of the mouse, to be followed by tumor implantation. Among these were the injection of acid, alkaline, or distilled water; the implantation of a small wad of glass wool; the injection of an air bleb, alcohol, turpentine, or histamine; and mechanical separation of the skin from the abdominal muscle wall. None but the last mentioned of these irritants produced consistent alterations in the growth of implanted tumor fragments. The type of inflammation induced by mechanical injury was, therefore, studied in detail in respect to its effect on tumor growth, and this effect comprises the main subject of this paper.

Procedure. A sharp-pointed 3-inch surgical scissors was inserted under the skin in the inguino-abdominal region and directed anteriad to a point about one-fourth inch from the axilla. With the position of the scissors' fulcrum lying at the point of original entry, the scissors was opened broadly so that the outer surfaces of its shears radically cleaved the skin from the underlying muscle tissue, without however puncturing the skin. Local bacterial infection was not evident in mice so treated, even when rigid surgical asepsis was not observed.

Groups of mice were so prepared for tumor implantation. Into some of these mice, fragments (approx. 2 mm^2) of sarcoma 180 were implanted immediately after the cleavage operation by inserting a trocar through the incision made by the scissors; the seeding fragment was deposited as close as possible to the center of the cleaved area. Fragments of comparable size and from the same donor tumor were implanted in the corresponding region of mice which had not been given the cleavage treatment; these served as controls.

Additional experiments were set up in which groups of cleavage-treated mice received tumor implants at various intervals after cleavage: at 24 hours after cleavage, at 48 hours, 72 hours, 6 days, and 12 days. In each instance tumors were implanted into parallel control groups of non-cleaved animals. The implanted fragment was allowed to grow for the usual 6 to 7 day period, at the end of which the tumorous growth was dissected out and wet-weighed. Before weights were taken, a puncture was made of the dissected tissue mass and exudative fluid was drained off. Rockland white male mice of 18-22 g were used throughout.

Results. For the first 4 days following fragment implantation into the cleaved subcutaneous area, tumor growth appeared to progress normally and was indistinguishable topographically from that of the controls. Between the 4th and 6th day after implantation a very marked swelling would become apparent in the area where the implant was deposited. Within 24 to 48 hours after the onset of this swelling, the experimental tumor appeared to attain a mass of from 5 to 10 times that of the control tumor (Plate I, Fig. 1 & 2). Upon dissection it was found that an extensive vesicle had developed throughout the cleavage area; and that a bleb of exudate had formed in close association with the growing tumor fragment (Plate I, Fig. 3). The vesicle fluid accounted for about half the total volume of the swelling. By the 9th day a hardening of the soft mass became evident. By the 12th day the edematous fluid was considerably replaced by the ingrowing tissue; the mouse in all such cases died before this process was complete, death usually occurring between the 12th and 15th day after implantation.

Animals implanted with tumor fragments immediately after cleavage exhibited the

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* The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council of the U. S. Public Health Service.

PLATE I.

Abdominal aspect of epilated mice to show relative growth of sarcoma 180 implant.
FIG. 1. Control tumor at 6-7 day stage (approx. ×2).
FIG. 2. Cleavage tumor at 6-7 day stage (approx. ×2).
FIG. 3. Cleavage tumor with skin removed and edematous bleb cut open (approx. ×4).

about 80% of the cleavage-treated mice, the remainder showing normal tumor growth. Occasionally an animal would lose its newly implanted fragment; such animals were rejected from the experiment.

Exudate withdrawn by syringe from the edematous bleb 6 days after tumor implantation was a pale pink serum, of pH 7.6, which when examined fresh or as a Wright's stain smear showed a sparse distribution of erythrocytes, some cells resembling lymphocytes and monocytes, and some polymorphonuclear leucocytes—a picture confirmed in Bouin-fixed hematoxylin-and-eosin sections of the tissue and bleb mass. At the interface of the serum and its encasing tissue (which appeared to be an intermixture of proliferating sarcoma cells and connective tissue elements) loose strands and masses of tumor cells extended inward, containing many cells in active mitosis (Plate II, Fig. 3, 4, & 5). The occurrence of such an inward infiltration was confirmed by examination of tissue fixed at 6, 8, 10, and 12 days after implantation. Very clearly, the induration of the mass palpably observed during the 8-12 day period was due to this ingrowth. Interspersed among the tumor cells at the growing fringe at the aforementioned interface were polymorphs, although their number was not such as to suggest suppuration, except in later stages when necrosis in the center of the vesicle often became more marked. Concomitant with tumor growth and the thickening of the vesicle wall, the primary tumor mass became undemarcably involved.

In further experiments a number of mice were subjected to the cleavage operation, but were not implanted with tumor tissue. Mice so prepared were killed at 1-day intervals following cleavage; the abdominal skin was incised and retracted so that observation could be made of the gross damage resulting from the cleavage. In most cases the inner surface of the cleaved skin showed some extravasation of blood, derived, obviously, from rupture of the larger vascular channels of the connective tissue during the scissors-cleavage procedure. The visible remains of such extravasation diminished after the 3rd day, and was no longer observable after the 6th. Restoration of fusion of the skin with the abdominal wall was not clearly complete until about the 5-6 day stage, as could be determined by the relative ease with which the skin could be withdrawn from the abdominal wall during dissection. Suppuration was never observed. The inner skin surface in the region cleaved showed some hyperemia, but never in conspicuous degree, and this hyperemia disappeared during the 3-6 day period. None of some 30 cleavage-control animals examined showed any discernible swelling of the area or any external evidence of edema.

The pronounced exudation observed in cleavage animals receiving tumor implants appeared to result from a specific interaction between the actively proliferating tumor tissue and the injured connective tissue substrate. Evidence of such a specificity was derived from an experiment in which fragments of muscle, tongue, intestine, spleen, and liver, instead of tumor tissue, were implanted into the cleaved area. In no instance did a palpable edema develop. The implanted non-tumorous tissue fragments were eventually resorbed without incident.

Comment. To interpret the interaction between tumor growth and the type of subcutaneous injury herein described, it may be of advantage to picture the events occurring under the mouse skin following cleavage injury. Moreover, it is necessary to consider what occurs when a tumor fragment is implanted in the usual fashion into non-injured subcutaneous tissue. In the latter case, the insertion of the trocar probably does not tear but merely distends the connective tissue. Thus the little damage that may occur is almost mechanically repaired at withdrawal of the trocar. Similarly, when the fragment is deposited, the connective tissue is stretched somewhat, but not

1

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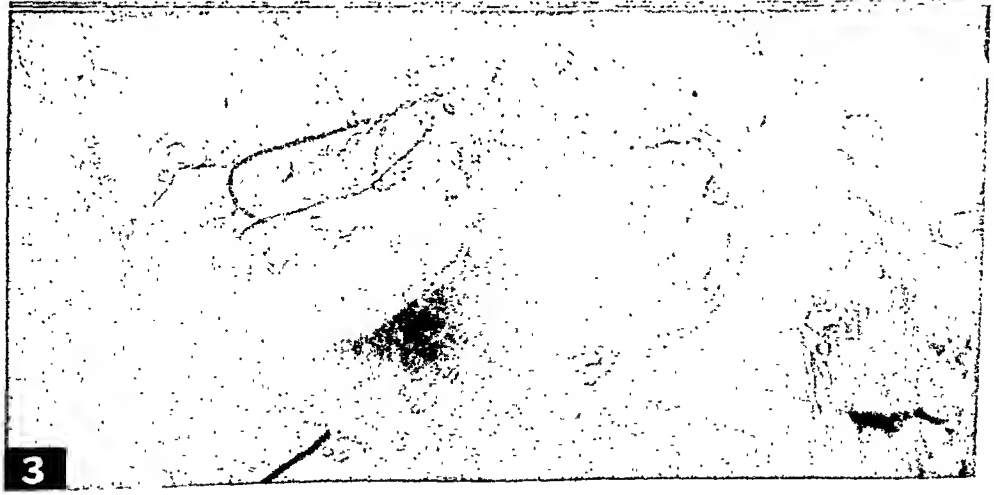
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PLATE II.

- FIG. 1. Shadowgraph of median sagittal section of 6-7 day cleavage tumor (approx. ×2.5).
- FIG. 2. Shadowgraph of median sagittal section of 9-10 day cleavage tumor (approx. ×2.5).
- FIG. 3. Area from Fig. 2, as indicated. Note large number of mitoses (high dry).
- FIG. 4. Area from Fig. 2, as indicated, to show mitotic sarcoma cell (oil immersion).
- FIG. 5. Interface between vesicle fluid and growing tumor surface, from Fig. 2, as indicated. Note mitoses (high dry).
- FIG. 6. Interface between vesicle fluid and growing tumor surface, from Fig. 1, as indicated. Note new capillary channels (high dry).

radically torn. During such normal implantation procedure there is little or no vascular rupture nor extravasation of blood.

The initial obvious reaction of the connective tissue to a tumor implant is one of classical imflammation, involving, hyperemia, appearance of polymorphs, and subsequently of mononuclear leucocytes.[1-3] The connective tissue area surrounding the new implant almost immediately becomes hyaline, and within a few days becomes infiltrated as a firm and expanding tumor mass. The original fragment does not change appreciably in size or shape (although its cells start undergoing degenerative changes almost at once after implantation) until about the 9th day when a fusion with surrounding necrotizing tissues renders its outlines and structure somewhat amorphous.

Following implantation of a tumor fragment, it is probable that phagocytic cells—as they do in a localized bacterial infection—attack the tumor cells at the periphery of the fragment. The tumor cells obviously win in the ensuing competition, for they proliferate vigorously and at the same time stimulate the growth of capillaries and, later, of larger vascular channels; in the meantime attracting large numbers of reticulo-endothelial elements. One of the fundamental differences between malignant and non-malignant tissue lies in the former's capacity to survive phagocytic or lytic onslaughts and to excite vascular proliferation in the substrate tissue.

Is the explosive edema described in this paper a tissue shock reaction due to the super-permeability of cleavage-inflamed capillary walls? If so, why is it elicited specifically by a tumor fragment and not at all by a normal tissue implant? It seems probable that cleavage injury, being vastly greater than that produced during simple trocar implantation, excites an excessive inflammatory and reparative response on the part of subcutaneous tissues involved. When into this dynamically unstable substrate a fragment of tumor tissue is implanted, the proliferative and stimulatory factors presumably released by the metabolizing tumor fragment find a richly vascularized and nutritious substrate in which to act or re-act.

The type or irritation produced by turpentine has been reported as not affecting carcinogenesis by benzpyrene,[4,5] which is consistent with our failure to note tumor growth enhancement by turpentine as well as by the variety of other irritating agents tested. Croton oil, on the other hand, has been reported as markedly augmenting chemical carcinogenesis, presumably by the exertion of a precarcinogenic action on skin tissues so treated.[6] Perhaps more cogent to the present discussion is the report that deep incisions made in the skin of mice previously treated by carcinogenic compounds showed localization to some extent of subsequent tumor growths.[6] It has been suggested, too, that cancer cells find a favorable environment in the region of healing wounds.[7] Whether such actions as these are related or analogous to the type of injury effect described in this paper is difficult to determine, due mainly to the variety of technics and the diversity of tumor types em-

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[1] Zahl, Paul A., and Drasher, M. L., *Cancer Research*, 1947, 7, 658.

[2] Zahl, Paul A., and Waters, L. L., PROC. SOC. EXP. BIOL. AND MED., 1941, 48, 304.

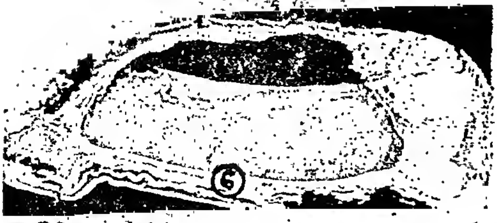
[3] Woglom, W. H., *The Study of Experimental Cancer*, New York City, 1913.

[4] Beck, S., *Brit. J. Exp. Path.*, 1938, 19, 319.

[5] Berenblum, I., *Cancer Research*, 1941, 1, 44, 807; *Arch. Path.*, 1944, 38, 233.

[6] Pullinger, B. D., *J. Path. and Bact.*, 1943, 55, 301.

[7] Kline, B. E., and Rusch, H. P., *Cancer Research*, 1944, 4, 762.



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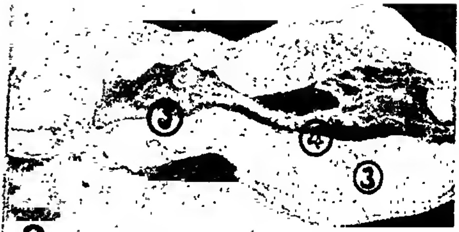

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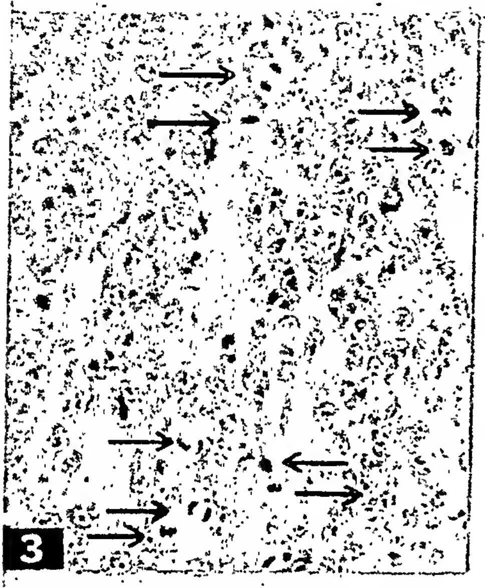

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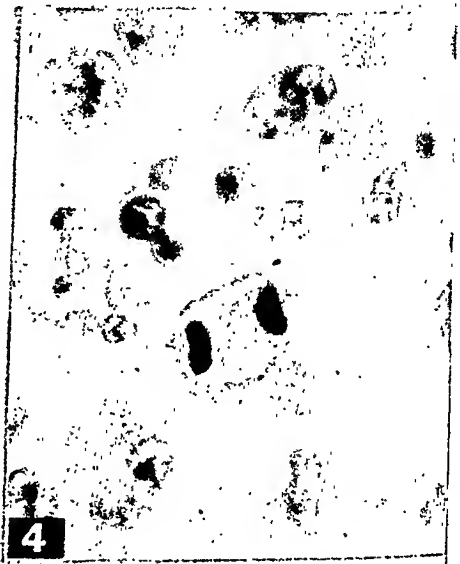

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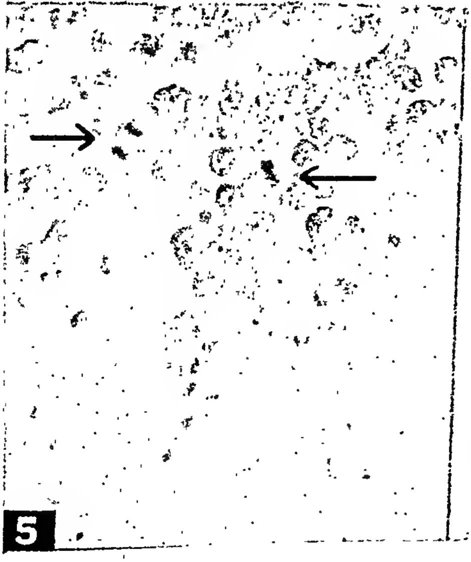

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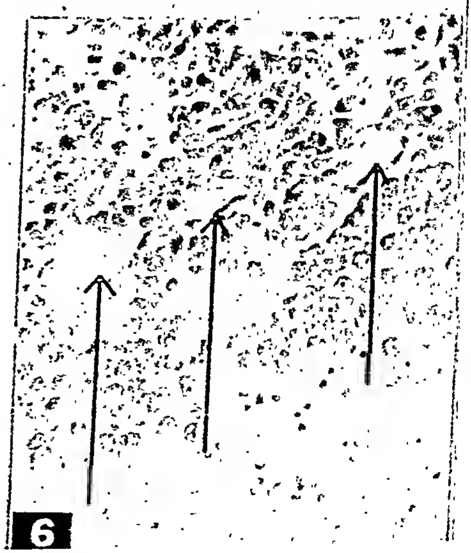

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of 50 to 100 mg/kg. Neostigmine methylsulfate was injected i-p in doses of 50 μg/kg.

Measurements of refractory period of nerve were made by delivering pairs of supramaximal stimuli through the same electrodes at various short intervals and determining the amplitude of the second response in relation to the first.

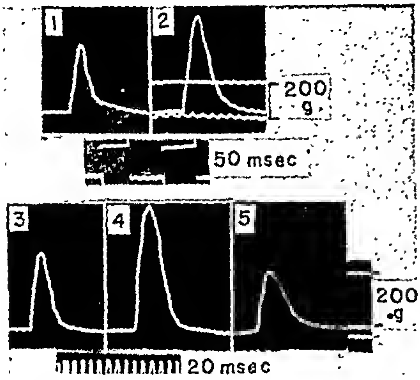

FIG. 1.
Myograms. 1. Control (indirect stimulation). 2. After 8 mg metrazol (resting tension 200 g). 3. Completely curarized muscle (direct stimulation). 4. After 20 mg DDT. 5. After 30 mg quinine diHCl (resting tension 35 g).

Results. Qualitatively, the effects of metrazol and of DDT were indistinguishable and are presented together. Both agents evoked in muscle a myotonic response which mimicked faithfully the functional and pharmacological characteristics of the myotonia occurring spontaneously in man and goat.[6] The myotonic response was seen as an increase in tension and duration of the twitch evoked by a single brief stimulus,[7] whether delivered indirectly through the sciatic nerve or directly into the curarised muscle (Fig. 1). The electrical basis of the augmented mechanical response was seen in the corresponding electromyograms, where the normal diphasic action potential was followed by a train of spikes (Fig. 2). Similar repetitive responses followed mechanical stimulation by tapping. Repeated stimulation at a rate of 12/min. produced a progressive waning of the myotonic response; the phenomenon of "warm-up". The isolated sciatic nerve studied *in situ* responded with a burst of spikes to a single stimulus following injection of either metrazol or DDT (Fig. 3).

Examples are given of the effect of agents which suppress the repetitive phenomenon: quinine (Fig. 1), Mg^{++} (Fig. 2) and Ca^{++} (Fig. 2 and 3). Accentuation of the myotonic effect by K^+ is seen in Fig. 2.

The effect of an injection of neostigmine, which itself did not produce repetition at the neuromuscular junction, was related to the amount of metrazol which had been given. After a small dose of metrazol (25 mg/kg), which produced a few random spikes, neostig-

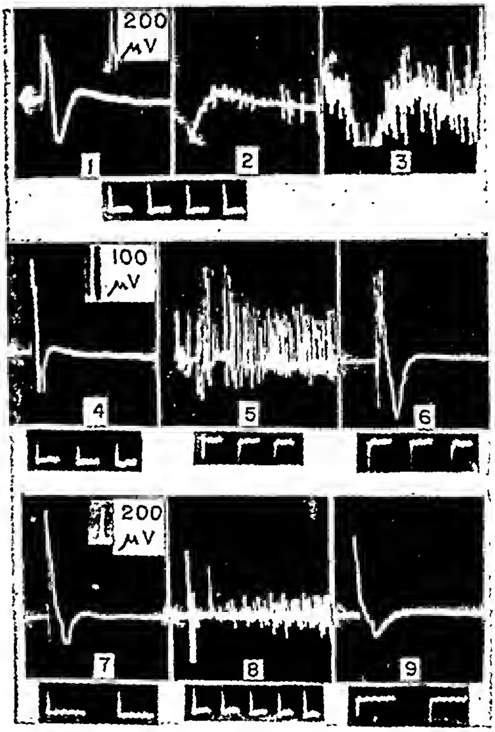

FIG. 2.
Electromyograms. 1. Control (indirect stimulation). 2. After 5 mg metrazol. 3. After 60 mg KCl. 4. Control (indirect stimulation). 5. After 20 mg DDT. 6. After 100 mg Ca gluconate. 7. Curarized control (direct stimulation). 8. After 20 mg DDT. 9. After 50 mg $MgSO_4$. All time scales = 100 msec.

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[5] Philips, F. S., and Gilman, A., *J. Pharmacol.*, 1946, 86, 213.

[6] Brown, G. L., and Harvey, A. M., *Brain*, 1939, 62, 341.

[7] Köllensperger, F. K., *Klin. Wchnschr.*, 1940, 19, 128.

ployed.

Summary. When a subcutaneous inflammation is induced in the mouse through mechanical injury, and when into such a traumatized site a fragment of experimental sarcoma is deposited, a striking edematous reaction ensues, accompanied by a conspicuous acceleration of tumor growth; these associated phenomena being well defined by the 4th to 6th day after implantation.

This effect is construed as a specific interaction between the neoplastic characteristics of the tumor fragment and the cellular and/or vascular sequences of subcutaneous inflammation. The effect does not occur when nonmalignant tissues are similarly implanted.

16896

Veratrinic Effects of Pentamethylenetetrazol (Metrazol) and 2,2-Bis (P-Chlorophenyl) 1,1,1 Trichloroethane (DDT) on Mammalian Neuromuscular Function.*

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(With the technical assistance of E. Leakins.)
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An extraordinarily diverse group of agents produces in excitable tissues a stereotyped, veratrinic effect: a single brief stimulus evokes a burst of repetitive responses.[1,2] Incomplete evidence indicates that the appearance of the veratrinic response in nerve fiber always is accompanied by a similar alteration in muscle fiber, and suggests that repetitive response is a general phenomenon which occurs in all excitable tissues exposed to veratrinic agents.

To explore further this general phenomenon, the study reported here was made of two agents which produce altered states of excitability. *Pentamethylenetetrazol* (metrazol) was selected because of its predominant effect on central neural activity and investigated for its effect on the peripheral neuromuscular unit. *2,2-bis (p-chlorophenyl) 1,1,1 trichloroethane* (DDT) was studied in the mammalian preparation because recent extensive investigations have demonstrated its potent veratrinic effect on the peripheral nerve of both Hexapoda and Crustacea.[3,4]

Methods. Details of the preparation and recording technics have been described.[2] Briefly, adult rats under pentobarbital anesthesia were used throughout. Isometric myograms were made from the triceps surae by means of resistance-wire strain gauges and recorded by means of a cathode ray oscillograph. Electromyograms were recorded through needles placed in the belly of the muscle. Electroneurograms were recorded from the sciatic-tibial nerve which had been isolated centrally and peripherally, Square-wave supramaximal stimuli were delivered to the sciatic nerve or directly to the curarised muscle. Metrazol was injected intraperitoneally (i-p) in doses of 25 to 100 mg/kg. DDT† was suspended in peanut oil-saline homogenate by means of lecithin (Asolectin)‡[5] and injected i-p in doses of 50 to 100 mg/kg. Neostigmine methylsulfate was injected i-p in doses of 50 µg/kg.

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* Work performed under a contract between the Office of Naval Research, U. S. Navy Department, and the Johns Hopkins University.

[1] Krayer, O., and Acheson, G. H., *Physiol. Rev.*, 1946, **26**, 383.

[2] Eyzaguirre, C., Folk, B. P., Zierler, K. L., and Lilienthal, J. L., Jr., *Am. J. Physiol.*, 1948, **155**, 69.

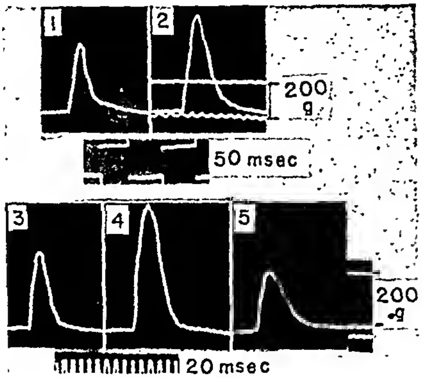
[3] Roeder, K. D., and Weiant, E. A., *Science*, 1946, **103**, 304.

[4] Welsh, J. H., and Gordon, H. T., *J. Cell. Comp. Physiol.*, 1947, **30**, 147.

† A twice-crystallized sample was kindly provided by the Medical Division, Army Chemical Center, Edgewood, Md.

‡ Generously provided by Associated Concentrates, Inc., Woodside, Long Island, N. Y.

Measurements of refractory period of nerve were made by delivering pairs of supramaximal stimuli through the same electrodes at various short intervals and determining the amplitude of the second response in relation to the first.



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Examples are given of the effect of agents which suppress the repetitive phenomenon: quinine (Fig. 1), Mg^{++} (Fig. 2) and Ca^{++} (Fig. 2 and 3). Accentuation of the myotonic effect by K^+ is seen in Fig. 2.

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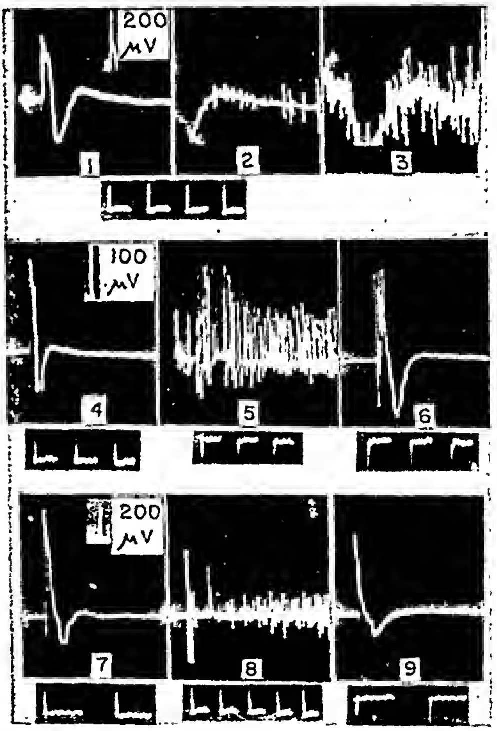

FIG. 2.

Electromyograms. 1. Control (indirect stimulation). 2. After 5 mg metrazol. 3. After 60 mg KCl. 4. Control (indirect stimulation). 5. After 20 mg DDT. 6. After 100 mg Ca gluconate. 7. Curarized control (direct stimulation). 8. After 20 mg DDT. 9. After 50 mg $MgSO_4$. All time scales = 100 msec.

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[5] Philips, F. S., and Gilman, A., *J. Pharmacol.*, 1946, 86, 213.

[6] Brown, G. L., and Harvey, A. M., *Brain*, 1939, 62, 341.

[7] Köllensperger, F. K., *Klin. Wchnschr.*, 1940, 19, 128.

mine accentuated the repetitive response. Conversely, after a larger dose of metrazol (50 mg/kg), which evoked intense repetition, the injection of neostigmine suppresed the usual response.

As a gauge of one phase of excitability, measurements were made in nerve of the rate of recovery of responsiveness, the relatively refractory period, before and after treatment with metrazol. The results of 2 experiments have been grouped and are presented in Fig. 4. Concurrent with the appearance of repetition after administration of metrazol, the nerve recovered more rapidly and the relatively refractory state subsided more abruptly. In addition, a minimal but recognizable period of supernormality appeared.

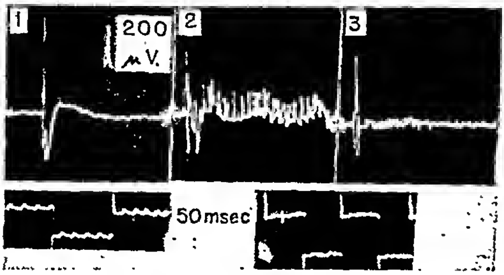

FIG. 3.
Electroneurograms. 1. Control. 2. After 10 mg DDT. 3. After 50 mg Ca gluconate.

Discussion. Evidence is accumulating slowly to support the hypothesis that agents which produce repetitive responses in one sort of excitable tissue induce generally the same altered state of excitability in other tissues of the same organism. An example of such a general effect is furnished by metrazol, which produces central rhythmic discharge and convulsions, and in the periphery evokes repetition in isolated nerve and striated muscle.

The same alteration of excitability resulting in repetition can be evoked in members of widely separated phyla. An example of this broad action is furnished by DDT which produces the same veratrinic response in the peripheral neuromuscular structures of both arthropods and mammals. DDT also fits into the group of substances which affect peripheral as well as several central structures.[8,9]

The changes which underlie the development of veratrinic repetition are obscure. The assumption that repetition occurs in a setting of exalted excitability is compatible with the demonstration of an accelerated rate of recovery demonstrated in nerve treated with metrazol or 2, 4-dichlorphenoxyacetate (2,4-D).[10] But the number of apparently unrelated agents which evoke repetition makes it difficult to construct an hypothesis encompassing all reported observations. A recent and attractive working hypothesis of repetition has emphasized the primary role of free and surface-bound calcium as a modulator of excitability in nerve.[11] Whether this engaging concept will explain repetition occurring in many tissues and under many varying conditions is an unanswered question.

The development of repetition in the peripheral neuromuscular unit results in a functional disorder which is indistinguishable from

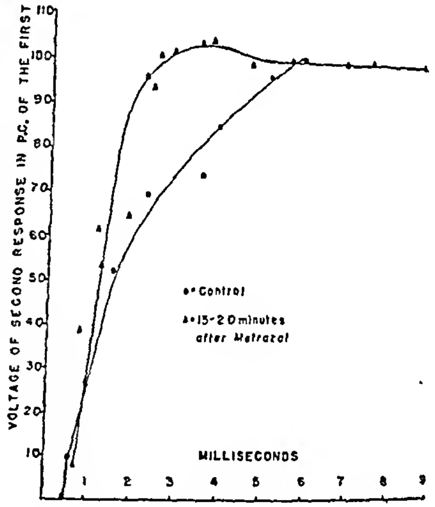

FIG. 4.
Relatively refractory period of nerve, measured before and after metrazol.

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[8] Crescitelli, F., and Gilman, A., *Am. J. Physiol.*, 1946, **147**, 127.

[9] Bromiley, R. B., and Bard, P., *Bull. Johns Hopkins Hosp.*, in press.

[10] Eyzaguirre, C., Jarcho, L. W., and Lilienthal, J. L., Jr., in preparation.

[11] Gordon, H. T., and Welsh, J. H., *J. Cell. Comp. Physiol.*, 1948, **31**, 395.

the myotonia occurring spontaneously in man and goat. There is no evidence, however, to establish the identity of the induced and the spontaneous phenomena, similar though they appear. Nevertheless, the demonstrated effect of veratrinic agents on both nerve and muscle suggests that in spontaneous myotonia an as yet undetected repetition may occur in nerve as well as in muscle.

Summary. 1. Both metrazol and DDT evoke a veratrinic response in mammalian nerve and muscle.

2. The characteristics of this response are indistinguishable from those of myotonia occurring spontaneously.

3. The veratrinic effect of repetitive responses to single stimuli is accompanied by a shortening of the relatively refractory period.

16897

Comparison of Atropine and Tripelennamine in Treatment of Peptone Shock in Dogs.

JOHN C. DAVIS, JR.* AND HANS O. HATERIUS.†

From the Department of Physiology, Boston University School of Medicine, Boston.

Dale and Laidlaw[1] first called attention to the close similarity existing between anaphylactic and peptone shock on the one hand and the type of shock produced by an injection of histamine on the other. Indeed, they postulated that histamine is the substance active in producing the classical signs of anaphylaxis. It was not until 1932, however, that their theory received substantiation, when Dragstedt[2] demonstrated the presence of a substance in blood and lymph in early stages of anaphylactic shock in dogs which resembled histamine chemically and physiologically. It is now generally accepted that histamine plays the major role both in peptone and in anaphylactic shock-like states.

Since the advent of potent anti-histaminic compounds, it has been demonstrated that all drugs which show a marked antagonism to histamine are capable of diminishing the severity of anaphylactic shock.[3] A number of observations, however, would indicate that some factor or factors other than histamine may be involved. For example, Went and Lissak[4] reported that the choline content of the isolated, sensitized heart of the guinea pig decreased following addition of antigen, and, moreover, that acetylation of the perfusion fluid evoked an acetylcholine-like reaction in leech muscle. Furthermore, the isolated, sensitized rodent heart slowed when antigen was added to the perfusing fluid. More recently, Farber and his co-workers[5] found that in 3 of 27 isolated, sensitized hearts (guinea pig) an acetylcholine-like substance was liberated to a perfusate containing physostigmine when the antigen was added. The *normal* heart failed to show the same response. Prior to this, Wenner and Buhrmester[6] reported the acetylcholine concentration in the blood of rabbits in anaphylactic shock to be 1:1,000,000 to 1:10,000,000, whereas measurable amounts were not detected in the blood of normal animals.

The relative importance of acetylcholine in the genesis of peptone and anaphylactic shock

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* The data in this paper were submitted to Boston University Graduate School in partial fulfillment of the requirements for the degree of Master of Arts.

† Deceased June 28, 1948.

[1] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910, **41**, 318.

[2] Dragstedt, C. A., and Gebauer-Fuelnegg, E., *Am. J. Physiol.*, 1932, **102**, 512.

[3] Loew, E. R., *Physiol. Rev.*, 1947, **27**, 542.

[4] Went, S., and Lissak, K., *Arch. f. Exp. Path.*, 1936, **182**, 509.

[5] Farber, S., Pope, A., and Landsteiner, E., Jr., *Arch. Path.*, 1944, **37**, 275.

[6] Wenner, W. F., and Buhrmester, C. C., *J. Allergy*, 1937, **9**, 85.

TABLE I.
Pulse Rates Following Administration of Peptone.

Pretreatment	Dose I.V. mg/kg	Exp. No.	Before administ.	During administ.	2-5 min. post-administ.
Atropine · SO_4	2	1	204	180	208
		2	188	168	200
		3	188	176	176
		4	188	152	148
		5	192	180	208
		Avg	192	171	188
Pyribenzamine · HCl	10	1	164	160	180
		2	168	140	180
		3	130	144	140
		4	228	192	268
		5	152	148	160
		Avg	168	157	186
Atropine · SO_4	2	1	160	144	150
and		2	190	140	208
Pyribenzamine	10	3	172	120	184
		4	160	152	188
		5	184	152	192
		Avg	173	142	184
Controls		1	172	160	192
		2	180	180	76
		3	184	144	96
		4	162	140	88
		5	160	140	72
		6	184	160	64
		Avg	174	154	98

has not been assayed, and the present report deals with attempts at a comparison of the beneficial effects of an antiacetylcholine agent and an antihistamine compound in peptone shock.

Methods. Mongrel dogs from a stock supply were anesthetized with sodium pentobarbital given by vein. The left carotid artery was exposed, cannulated, and connected with a U-tube mercury manometer modified for use with the electrical recording system described by Maison and Haterius.[7] Pulse rates were obtained by palpation. All drugs were administered via the femoral vein. Atropine sulfate, 2.0 mg/kg and Tripelennamine (pyribenzamine),‡ 10.0 mg/kg either alone or in combination were used as prepeptone medication. These were administered as 1.0% solutions. Ten to 15 minutes were allowed after the administration of pyribenzamine before peptone was given. Five cc (1.0 g) per kilo of a 20% solution of Witte's peptone was then injected intravenously as rapidly as possible; the time required for the injection was 1.5 to 3 minutes depending upon the total volume used.

Results. Mortality: Of 6 controls, receiving only peptone, 5 died within 5 to 11 minutes after beginning the injection. Of 6 dogs pretreated with atropine, 5 survived; of 5 receiving pyribenzamine all survived, and of 5 receiving both pyribenzamine and atropine there were no deaths. The statistical significance between control and pretreated dogs is great ($P = <.01$) but none exists between methods of pretreatment.

Pulse rates: During the peptone injection, the heart rate in most of the animals slowed 4 to 52 beats per minute from the initial rate. Within 2-5 minutes after the beginning of injection, the pulse rates of those dogs which were to survive increased to a level greater than that prior to administration of peptone. The controls developing fatal shock did not exhibit this increase, but rather showed a constant decrease in rate until death. The changes in pulse rates are apparent from Table I.

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[7] Maison, G. L., and Haterius, H. O., *J. Assn. Am. Med. Coll.*, 1947, **22**, 200.

‡ The Pyribenzamine was kindly supplied by Ciba Pharmaceutical Products, Inc.

Blood pressure: All of the dogs, regardless of the type of pretreatment, showed an initial precipitous fall in blood pressure to about 30 mm Hg beginning within 15 to 30 seconds after commencing the peptone injection. Fig. 1 is an example of this initial drop in blood pressure. Continuation of the injection evoked a further fall of only 5 to 10 mm in the treated animals, while the controls showed a progressive decline to zero. In a majority of the surviving animals the blood pressure began to rise in 4 to 10 minutes, reached a peak in 9 to 16 minutes, declined a few millimeters and then began a secondary more gradual rise within 20 to 30 minutes, reaching a plateau in about one hour. (All time intervals given refer to the time elapsed from the beginning of the peptone injection.) This initial rise, as well as the increase in pulse rates to greater than pre-peptone levels, is probably indicative of a generalized sympathetic discharge occurring in response to the profound hypotension. Six animals did not show the initial peak, but exhibited a slow continuous rise to the final plateau. Otherwise they were similar to the remaining animals. A typical example of the blood pressure changes for each of the 3 methods of pre-treatment is given in Fig. 2.

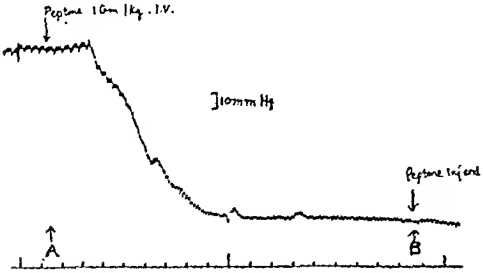

FIG. 1.

A typical example of the effect upon the blood pressure produced by the intravenous injection of peptone, 1.0 g/kg. Injection begun at A, completed at B. Time interval—6 seconds. Pressure at A, 140 mm Hg; pressure at B, 28 mm Hg.

As pyribenzamine potentiates the response to epinephrine,[8] the blood pressure level at 25 minutes post-peptone, (at which time the pressure was at a plateau or just beginning its secondary rise) was used as an indication of the degree of protection afforded by the drugs, since this would minimize the effects of sympathetic discharge which were probably greatest somewhat earlier coincident with marked hypotension. Twenty-five minutes were arbitrarily chosen, because with further recovery the differences between the methods used became less marked. The blood pressure changes are shown in Table II.

None of the 3 methods of treatment prevented the initial fall in blood pressure. Both the height of the initial peak and the pressure at 25 minutes were greater in those treated with pyribenzamine alone than in those receiving atropine alone. In the dosages used all 3 methods of treatment protected dogs from death due to peptone. On the basis of blood pressure levels at 25 minutes after the adminis-

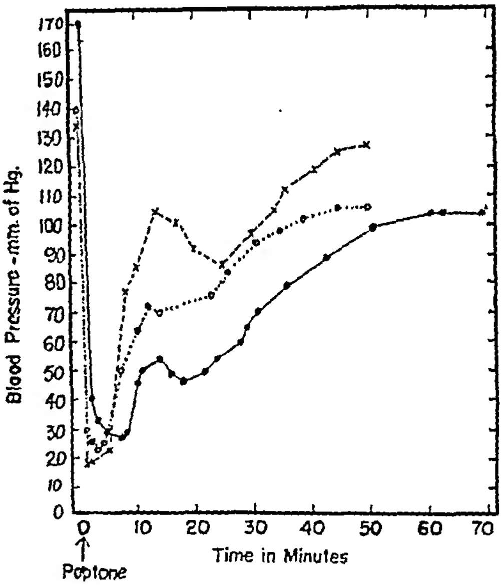

FIG. 2.

Typical blood pressure changes occurring after administration of peptone, 1.0 g/kg body weight. Pretreatment:

●——● Atropine sulfate, 2.0 mg/kg; Exp. No. 2.

x– – – –x Pyribenzamine • HCl, 10.0 mg/kg; Exp. No. 2.

○····○ Atropine sulfate, 2 mg/kg, and Pyribenzamine • HCl, 10 mg/kg. Exp. No. 5.

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[8] Sherrod, T. R., Loew, E. R., and Schloemer, H. F., *J. Pharm. and Exp. Therap.*, 1947, 89, 247.

TABLE II.
Blood Pressure Following Administration of Peptone.

Pretreatment	Dose I.V. mg/kg	Exp. No.	Blood pressure (mm Hg)		
			Before administ.	2 min. after administ.	25 min. after administ.*
Atropine • SO_4	2	1	138	30	45
		2‡	170	34	56
		3	134	18	44
		4	112	18	10
		5	136	22	96
		Avg	138	24	50
Pyribenzamine • HCl	10	1	126	52	112
		2‡	142	20	88
		3	128	26	104
		4	152	36	76
		5	122	28	88
		Avg	134	32	94
Atropine • SO_4 and Pyribenzamine • HCl	2 10	1	80	28	106
		2	68	10	44
		3	144	24	56
		4	136	22	70
		5‡	136	24	80
		Avg	113	22	71
Controls		1	126	10	42†
		2	138	10	—
		3	150	18	—
		4	146	24	—
		5	140	16	—
		6	120	10	—
		Avg	137	15	—

* *Statistical evaluation of atropine vs. pyribenzamine (t-test) shows P = .02.*

† Only one of 6 controls survived, 5 died in 5 to 11 *minutes as* blood *pressure reached* 0 mm Hg.

‡ Data plotted in Fig. 2 as typical examples of each method of treatment.

tration of peptone, pyribenzamine afforded significantly better protection than atropine (P = .02). A combination of the two did not appear to afford better protection than pyribenzamine alone, as the rate of recovery from the hypotensive state was no greater in those receiving both drugs than in those receiving only pyribenzamine.

Discussion. If acetylcholine plays a role in the genesis of anaphylactic and peptone shock, pretreatment with a substance that blocks its action should lessen the severity of reaction. As long ago as 1910 Auer[9] reported atropine to be of value in anaphylactic shock, and obtained a 72% survival in guinea pigs pretreated with atropine before reinjection of the sensitizing serum, as compared with 25% survival in non-atropinized controls. Gross,[10] in finding 0.3 g/kg of Witte's peptone to be the MLD in dogs, reported atropine (0.2 mg/kg) would prevent death in all dogs subjected to 0.5 g/kg of peptone. Atropine in this dosage failed to protect against the lethal effects of 0.6 g/kg. The number of animals used was not stated.

On the other hand, in a report by Vallery-Radot, Mauric and Holtzer,[11] atropine in doses of 0.004 to 0.5 mg/kg did not prevent anaphylactic shock in rabbits. Of 7 animals, 2 (on a dosage respectively of 0.5 mg and 1.0 mg) did not exhibit shock. Despite this the authors concluded that atropine was ineffective in preventing this type of shock. Danielopolou,[12] however, reported atropine to hinder, and eserine to aid, the development of anaphylaxis. Dosages were not given.

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[9] Auer, J., *Am. J. Physiol.*, 1910, **26**, 439.

[10] Gross, E. G., *J. Pharm. and Exp. Therap.*, 1926, **30**, 351.

[11] Vallery-Radot, P., Mauric, G., and Holtzer, A., *Compt. rend. Soc. de Biol.*, 1943, **137**, 18.

[12] Danielopolou, D., *Acta. Med. Scandinav.*, 1944, **118**, 22.

In the present instance, the failure to prevent the initial fall in blood pressure is consistent with the findings of Yonkman, Hays and Rennick[13] in anaphylactic shock. They reported, however, that the heart rate is apparently not increased during the hypotensive phase in anaphylaxis following pretreatment with pyribenzamine—a circumstance with which our results in the case of peptone shock are not in agreement.

One difficulty to be overcome before accepting the hypothesis that acetylcholine plays a role in the production of peptone shock is that, as yet, no drug is available which exhibits antagonism to acetylcholine without possessing some antihistaminic action. Similarly all antihistaminic drugs show a certain degree of antiacetylcholine activity. Various degrees of specificity are possible however. Thus pyribenzamine is 250 times as potent as atropine against histamine as tested by the aerosol method of producing histamine shock in guinea pigs.[14,15] Atropine is much more effective as an anticholinergic drug than is pyribenzamine. In our experience 10.0 mg/kg of pyribenzamine exerted only moderate vagal blockage, as compared with 0.05 mg/kg of atropine required for vagal blocking in the dog.[16]

To protect 100% of sensitized guinea pigs from lethal anaphylaxis, 0.3 mg/kg of pyribenzamine must be given.[15] The exact dosage required to protect dogs has not been determined, but it seems to be somewhat greater. Thus the protective action of 2.0 mg/kg of atropine in peptone shock can hardly be attributed to its antihistaminic properties. It seems much more likely that the benefit derived is a result of its anticholinergic activity.

Although it is generally accepted that histamine plays an important role in anaphylactic and peptone shock, it is possible that acetylcholine is also involved to some extent. The relative importance of histamine and acetylcholine remains to be determined more precisely. More quantitative comparisons between the antihistaminics and anticholinergic drugs are necessary before the question can be resolved, but the greater degree of protection afforded by pyribenzamine than by atropine, as shown in these experiments by the blood pressure changes, is evidence that while histamine plays the major role in the production of peptone shock, apparently acetylcholine is also concerned.

Conclusions. 1) One gram/kg body weight of Witte's peptone administered intravenously in a 20% solution, proved fatal in dogs anesthetized with sodium pentobarbital.

2) Atropine sulfate. 2.0 mg/kg prevented death from peptone.

3) Pyribenzamine, 10.0 mg/kg, afforded better protection than atropine as determined by rapidity of recovery of blood pressure.

4) The rapidity of recovery of blood pressure was not greater in those animals receiving both pyribenzamine and atropine than in those receiving pyribenzamine alone.

5) These findings support the theory that acetylcholine is concerned in the production of peptone shock, although histamine plays a more important role.

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[13] Yonkman, F. F., Hays, H. W., and Rennick, B., *Fed. Proc.*, 1944, 4, 144.

[14] Halpern, B. N., *Arch. Internat. de Pharmacodyn. et de Therap.*, 1942, 68, 339.

[15] Mayer, R. L., *J. Allergy*, 1946, 17, 153.

[16] Sollmann, T. H., and Hanzlik, P. J., *Fundamentals of Experimental Pharmacology*, J. W. Stacey, Inc., San Francisco, 1939.

16898

Influence of Secretin and Insulin on Pancreatic Secretion in Healthy Human Subjects.

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The intravenous administration of highly purified secretin is known to stimulate the secretion of pancreatic juice which is low in both organic material and enzymatic activity. On the other hand vagus stimulation results in the secretion of pancreatic juice with a high enzyme concentration.[1] Central vagus stimulation induced by insulin hypoglycemia has been shown to be without effect on the secretion of fluid by the pancreas in both man[2] and the unanesthetized dog[3] providing adequate precautions are taken to exclude the acid gastric contents from the intestine. However, less consistent have been the studies on the influence of insulin hypoglycemia on the secretion of enzymes by the pancreas. Both inhibition[4,5] and excitation[6,7] of enzyme secretion have been reported.

Methods. The subjects comprising this study were 22 men and women who were either volunteers or clinic patients with no evidence of either gastro-intestinal or metabolic disease. About 15 to 18 hours after the previous meal the stomach and intestine were intubated with a single double-lumen tube of the type described by Agren and Lagerlof.[8,9] Exact location of the tube was always verified by fluoroscopy. Separate gastric and duodenal samples were collected by continuous aspiration at a negative pressure of 30 to 50 mm Hg.

The secretin used was prepared either in our laboratory or by Wyeth Incorporated by the procedure of Friedman and Thomas.[10] For the purpose of this study it is important to note that no evidence for the presence of pancreozymin[11] in this secretin preparation was found. The secretin had no effect on the blood sugar level, and did not stimulate either emptying of the gall-bladder or the secretion of intestinal juice.*

Following collection of a basal sample during a 20 minute control period the secretin was administered intravenously at a dosage level of 1.1 clinical units per kilogram body weight. Continuous collection of separate gastric and intestinal contents was carried out for the next 60 minutes, the samples being divided into the fractions obtained at the end of 10, 20, 40 and 60 minutes. A second standard dose of secretin was then given and collection of gastric and duodenal contents continued for the next hour. In one series of patients the second intravenous dose of secretin was combined with crystalline insulin, 0.1 unit per kilogram body weight. In some experiments a third injection of either secretin alone, or of secretin combined with insulin in the doses stated, was made at the end of the second hour and collection of digestive

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[1] Babkin, B. P., *Secretory Mechanism of the Digestive Glands*, New York, Hoeber, 1942.

[2] Frisk, A. R., and Welin, G., *Acta med. Scand.*, 1937, **91**, 170.

[3] Scott, V. B., Collingnon, U. J., Bugel, H. J., and Johnson, G. C., *Am. J. Physiol.*, 1941, **134**, 208.

[4] Baxter, S. G., *Quart. J. Exp. Physiol.*, 1932, **21**, 355.

[5] Hebb, C. O., *Quart. J. Exp. Physiol.*, 1937, **26** 339.

[6] Lagerlof, H., and Welin, G., *Acta med. Scand.*, 1937, **91**, 397.

[7] Thomas, J. E., and Crider, J. O., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 27.

[8] Agren, G., and Lagerlof, H., *Acta med. Scand.*, 1936, **90**, 1.

[9] Lagerlof, H., *Pancreatic Function and Pancreatic Disease Studied by Means of Secretin*, New York, Macmillan, 1942.

[10] Friedman, M. H. F., and Thomas, J. E., to be published.

[11] Harper, A. A., and Raper, S. S., *J. Physiol.*, 1943, **102**, 115.

* We are indebted to Dr. E. S. Nassett for the assay for enterocrinin.

TABLE I.

	1st hr Secretin			2nd hr Secretin		
	cc	Output	Conc. K	cc	Output	Conc. K
Highest	213	48140	.434	253	59720	.477
Lowest	82	7870	.072	127	9982	.072
Avg	143	22658	.158	186	29244	.157
Increase, %				30.1	29.1	0
	Secretin			Secretin + insulin		
Highest	283	67354	.238	287	57680	.288
Lowest	94	9580	.056	119	16070	.093
Avg	163	24910	.153	195	37812	.194
Increase, %				19.2	51.8	26.7

fluids continued during the third hour. Blood samples for sugar determinations were taken immediately before the combined injection of insulin and secretin and at 30 minutes and 60 minutes after the injection. All experiments involving the use of insulin were terminated by giving the subject dextrose solution through the gastric tube. Bicarbonate was determined by the titrometric procedure of Van Slyke.[12] Lipase concentration was determined on a tributyrin substrate by a phototurbidometric method,[13] and expressed in terms of percent standard substrate hydrolyzed by 0.1 cc of duodenal fluid in 20 minutes. Amylase concentration was determined by the picric acid method of Myers, Free and Rosinski.[14] Trypsin concentration was determined on an albumen substrate by the turbidometric method of Riggs and Stadie[15] as modified by Friedman.[16] Blood sugar concentration was determined by the Folin-Wu procedure.

Results. Following the injection of secretin the secretion of pancreatic juice reached its maximum rate during the second ten minute period and amounted to 82 to 213 cc (average 143 cc) during the hour (Table I). The concentration of enzymes was also greatest during the second 10 minute period but decreased thereafter at a more rapid rate than did the volume. In some instances the fluid recovered from the intestine during the last 20 minutes was pratically free from trypsin while amylase and lipase were still present in measurable concentrations.

A second dose of secretin in 22 experiments yielded from 127 to 253 cc (average 186 cc) of intestinal content during the hour, with the maximum rate of secretion usually occurring during the first 20-minute period. The secretory rate fell less rapidly during the hour following the second injection of secretin than during the hour following the first injection. The concentration of enzymes following the second secretin injection usually reached a maximum during the first 10 minute period. In all except 3 of the 22 experiments the concentration of enzyme following the second dose of secretin was less than that following the first dose (Table I).

In 17 other experiments the second secretin injection was combined with the injection of insulin. The blood sugar levels before insulin ranged from 66 to 93 mg %, 30 minutes after the administration of insulin from 23 to 48 mg %, and at the end of one hour from 30 to 105 mg %. In only one patient did the blood sugar level at the end of the hour exceed the initial value. During the first hour, when secretin alone was given, the volume secreted ranged from 94 to 283 cc (average 163 cc) while during the second hour, when insulin was combined with the secretin, the volume ranged from 119 to 287 cc (average 195 cc). The rate of secretion following the administration of insulin combined with secretin was thus not different from that occurring following a second dose of secretin alone. However, in contrast with experiments in which secretin alone was given, in each of these experiments the concentration of enzymes secreted during the second hour was higher than during the first hour. The average increase in output of enzymes was 51.8% (Table I).

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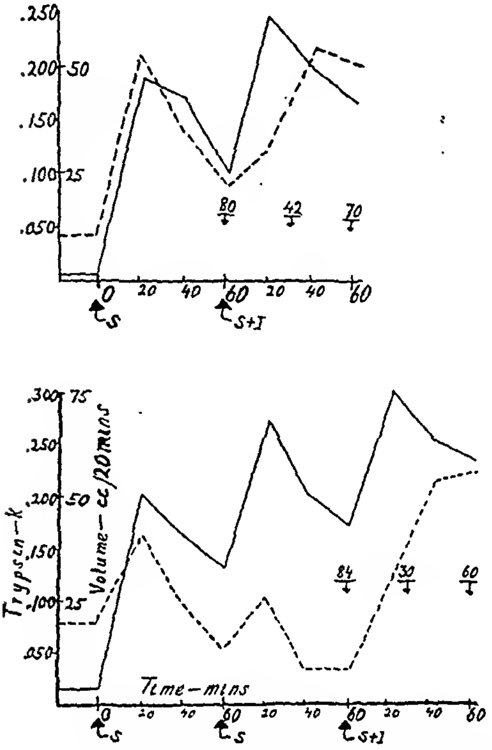
[12] Van Slyke, D. D., Stillman, E., and Culler, G. E., *J. Biol. Chem.*, 1919, **38**, 167.

[13] Friedman, M. H. F., to be published.

[14] Myers, V. C., Free, A. H., and Rosinski, E. E., *J. Biol. Chem.*, 1944, **154**, 39.

[15] Riggs, B. C., and Stadie, W. C., *J. Biol. Chem.*, 1943, **150**, 463.

[16] Friedman, M. H. F., *Gastroenterol.*, 1947, **8**, 527.



Mr. L. L., psychoneurosis, no gastrointestinal disease demonstrated. Underscored figures in each graph represent mg % blood sugar before and after insulin. Solid line shows volume, dotted line shows trypsin concentration.

FIG. 1a (upper) Pancreatic response to a dose of secretin (S) followed one hour later by a dose of secretin combined with insulin (S + I).

FIG. 1b (lower). Pancreatic response to 3 hourly injections of secretin (S), the last one being combined with insulin (S + I).

In 6 experiments 3 hourly injections of secretin were given. In 3 of these experiments the third secretin injection was combined with insulin. Three consecutive hourly injections of secretin alone resulted in a fairly steady increase in rate of secretion with a progressive decrease in the concentration and total output of enzymes. Combination of insulin with the third secretin injection had little influence on the rate of secretion but resulted in a very definite increase in the enzyme output (Fig. 1).

Discussion. The intravenous administration of secretin to the healthy fasting subjects evoked the typical pancreatic response attributed to the hormone. After a second dose of secretin the volume of juice secreted was greater than after the first dose, probably due to the cumulative effects of both secretin injections. After each injection of secretin the concentration of enzymes gradually diminished following an initial transitory rise. Since secretin is believed to excite active secretion of bicarbonate and water only, but not of enzymes, the initial transitory increase in enzyme concentration has generally been attributed to the washing out of the enzyme from the acinar cells and ducts by the flow of water. According to some investigators[17] a constant synthesis of protein material would make available new enzyme substances which would be similarly washed out by the flow of water that is put into operation by a second dose of secretin. Experimental evidence for this process, however, is still wanting.

Another explanation has been advanced by Thomas[18] and recently by Wang *et al.*[19] An increase in the dose of secretin, as well as a new injection of a submaximal dose of secretin, as was done in our experiments, may bring previously resting secretory elements into activity. This would result in the initial transient increase in enzyme output after each secretin injection.

Compared to the effects of secretin alone, the addition of insulin to the secretin did not influence the volume response. However, the output of enzymes by the pancreas secreting in response to secretin was increased by about 60% when a hypoglycemic state was induced by means of insulin. It is well-known that vagus excitation may stimulate the secretion of pancreatic juice of high enzyme concentration, and that insulin hypoglycemia may excite vagus centers. The latter has been proved abundantly by studies on gastric secretion and motility. It may be assumed that the excitatory effect of insulin on the secretion of pancreatic enzymes is due to a generalized hypoglycemic excitation of visceral centers, including those of the vagus.

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[17] Komarov, S. A., Langstroth, G. O., and McRae, D. R., *Canadian J. Research*, 1939, **17**, 113.

[18] Thomas, J. E., *Fed. Proc.*, 1942, **1**, 261.

[19] Wang, C. C., Grossman, M. I., and Ivy, A. C., *Am. J. Physiol.*, 1948, **154**, 358.

Summary. Healthy human subjects were given 2 or 3 hourly intravenous injections of secretin. In some of the experiments insulin was given intravenously during the course of the pancreatic secretion evoked by the secretin. It was found that during insulin hypoglycemia the concentration and output of pancreatic enzymes was increased but that the volume of secretion was not influenced.

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Heat Stability of Hemagglutinin of Various Strains of Newcastle Disease Virus.*

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Determination of the heat stability of the hemagglutinin was selected as the first method of approach to the problem of characterizing the antigenically homologous strains[1] of Newcastle (NDV) virus. A method of identifying strains was deemed necessary for proposed studies of virulence and immunogenicity of the virus. For purpose of survey, 24 strains of virus obtained from widely distributed points in North America and Europe were cultured in embryonating eggs and the stability of the hemagglutinin (HA) to heating at 56°C determined.

The heat stability of the influenza hemagglutinin[2] has been extensively studied. It was the demonstration by Salk[3] of differences in heat stability of the influenza strains which influenced our choice of approach. Scott and Lauffer[4] described the inactivation of hemagglutinin of influenza virus as a first order reaction by assuming a multicomponent system. The demonstration that the site of inactivation of the hemagglutinin and the site of infectivity for embryos were independent was, therefore, not surprising.[5] The Henles[6] have shown that with heat and ultraviolet treatment, certain properties of influenza virus can be arranged in a descending order of susceptibility to inactivation. The capacity to infect embryos was lost first, then, in order, the ability to interfere with infection, to elute from agglutinated red blood cells, to agglutinate red blood cells, and subsequently, the ability to fix complement in the presence of immune sera. Antigenicity could not always be fitted into this order, but sometimes it persisted until after the hemagglutinin had been destroyed. With certain strains of influenza virus Salk[7] demonstrated the protec-

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Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[1] Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. E., *Am. J. Vet. Res.*, 1946, **7**, 289.

[2] Burnet, F. M., *Aust. J. Exp. Biol. and Med. Sci.*, 1942, **20**, 81.

[3] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 134.

[4] Scott, E. M., and Lauffer, M. A., *Arch. Biochem.*, 1946, **11**, 185.

[5] Lauffer, M. A., Carnelly, H. L., and MacDonald, E., *Arch. Biochem.*, 1948, **16**, 321.

[6] Henle, W., and Henle, G., *J. Exp. Med.*, 1947, **85**, 347.

[7] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 140.

TABLE I.
Description of Strains.

Place of isolation	Date of isolation	Donor	Source animal	Code
New Jersey	1944	F. R. Beaudette	Chicken	N. J. (NJ-KD)
?	?	F. R. Beaudette	?	Beaudette vaccine (FRB)
New York	45	P. P. Levine	Chicken	N. Y. Levine (NYL)
New York	46	J. Fabricant	Turkey	Mayer turkey (NYT)
Massachusetts	45	C. A. Brandly	Chicken	Huntington Lab (4F)
Iowa	46	M. A. Hofstadt	Chicken	23 E 219 (HOF)
Michigan	46	C. H. Cunningham	,,	46-967 (MIC)
Missouri	47	H. C. McDougle	,,	31747 (31747)
Kansas	48	L. D. Bushnell	,,	Leavenworth (KL)
Kansas	48	L. D. Bushnell	,,	Manhattan (KM)
Wisconsin	46	Field	,,	Lancaster 1660 (WL)
Wisconsin	48	Field	Turkey	Henry 24156 (HHT56)
Minnesota	47	B. S. Pomeroy	,,	(TM)
California	44	J. R. Beach	Chicken	(11914)
Canada	47	R. V. L. Walker	,,	Allen M4947 (ALL)
,,	47	R. V. L. Walker	,,	Berwick 29507 (BER)
,,	47	R. V. L. Walker	,,	Cleroux M4969 (CLE)
,,	47	R. V. L. Walker	,,	Herr M5004 (HER)
,,	47	R. V. L. Walker	,,	Labello M4970 (LAB)
,,	47	R. V. L. Walker	,,	McVey M5016 (McV)
,,	47	R. V. L. Walker	,,	Watford No. 5 (WAT)
,,	47	R. V. L. Walker	Turkey	Peerless M4945 (TP)
England	33	Min. of Agric.	Chicken	Hertfordshire (E)
Italy	45	U. S. Army	,,	Milano (M)

tive action of traces of formalin to the denaturation process. Francis[8] reported the divergence of the capacity to show inhibition in the presence of immune sera when the hemagglutinin was heated.

Methods. For ease of comparison and for the sake of simplicity the methods used follow those for similar work with influenza virus insofar as the properties of Newcastle virus permit.

Virus material from an early passage of each strain (less than 10 egg passages) was utilized to avoid changes which may develop in the character of the strain on prolonged cultivation of Newcastle virus in the embryonating egg.[1,9-12] Only freshly harvested virus or material held for less than one month at —20°C was used for determinations. Allanto-amnionic fluids become less stable to heat treatment after storage at 4°C, but no significant changes were observed after storage at —20°C for the periods and strains studied.

Clear allanto-amnionic fluids from moribund or dead embryos were sealed in 5 ml lyophilizing vials by drawing out the necks of the vials in a flame. The vials were submerged in a constant temperature water bath at 56 ± 0.5°C, the water being agitated constantly with an electric stirrer. Vials were removed at selected intervals and immediately chilled in ice water. Pooled allanto-amnionic fluids from four to eight eggs were used for all determinations. Heat stability data were obtained on at least two pools and in most instances four or five pools of each strain. Virus pools of a given strain, but not necessarily individual egg fluids of a given strain, gave closely reproducible results. Reasons for individual egg variations have been explored, and with certain precautions observed in har-

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[8] Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

[9] Iyer, S. G., and Dobson, N., *Vet. Rec.*, 1941, **53**, 381.

[10] Iyer, S. G., and Hashmi, Z. A., *Indian J. Vet. Sci.*, 1945, **15**, 155.

[11] Winslow, N. S., 1947. Thesis submitted for degree of Master of Science, University of Wisconsin.

[12] Beaudette, F. R., Proc. 47th Ann. Meet. U. S. Livestock San. Assoc., 1943, 122.

TABLE II.
Heat Stability of the Hemagglutinin at 56°C.

Strain	Minutes* 0	15	30	60	120	240	480
NJ-KD	1280	0	0	0	0	0	0
FRB	1280	0	0	0	0	0	0
NYL	1280	20	0	0	0	0	0
NYT	1280	10	0	0	0	0	0
4F	1280	160	10	0	0	0	0
HOF	1280	160	20	0	0	0	0
MIC	1280	640	40	0	0	0	0
31747	1280	320	40	0	0	0	0
KL	1280	1280	1280	1280	320	160	0
KM	1280	1280	1280	640	320	5	0
WL	1280	40	0	0	0	0	0
HHT56	1280	0	0	0	0	0	0
TM	1280	1280	640	640	160	10	0
11914	1280	320	160	10	0	0	0
ALL	1280	1280	1280	640	320	160	0
BER	1280	1280	1280	640	320	20	0
CLE	1280	1280	1280	640	80	20	0
HER	1280	1280	1280	1280	640	160	0
LAB	1280	1280	640	640	320	10	0
McV	1280	640	320	160	20	0	0
WAT	1280	1280	640	40	0	0	0
TP	1280	1280	1280	640	320	40	0
E	1280	160	20	0	0	0	0
M	1280	1280	1280	640	80	20	0

* Data obtained at different time intervals (see text) are not included in table.

vesting fluids, close correlation can be obtained among individual egg fluids containing virus of a given strain.

The virus pools ranged between pH 7.9 and pH 8.2. There was no relationship between pH of the fluids on harvest and stability of the virus in the fluid to heating.

The hemagglutination test procedure substantially followed that outlined by Salk.[13] A 0.2 ml volume of virus was added to 0.8 ml of saline in agglutination tubes (14.5 × 75 mm). Five-tenths of a milliliter of this 1:5 dilution was transferred to 0.5 ml of saline, thoroughly mixed, and similar transfers repeated until a 1-1280 dilution was obtained. Twenty-five hundredths milliliters of 1% fowl red blood cells was added to all tubes and the rack shaken well. The hydrogen ion concentration of the virus-saline-red cell mixtures ranged from pH 7 to 8. The test was incubated at room temperature of 25 to 30°C and read from 15 to 45 minutes later,[14] the period of reading being determined by the controls. The endpoint was considered to be a definite one plus agglutination. The effect on titers of differences in the agglutinability of red cells from one fowl to another was considerably resolved by using the highly agglutinable cells of a single fowl as a standard for all tests.[14]

Experimental. A description of the 24 strains is given in Table I. The localities of isolation are roughly grouped into 5 regions, eastern United States, the middle western United States, western United States, Canada, and Europe. The dates of isolation range from 1933 to 1948, a period of 15 years. Four of the strains were obtained from turkeys and 20 from chickens. The abbreviated code given in parentheses following the complete strain designation is used in the text and accompanying figures.

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[13] Salk, J. E., *J. Immunol.*, 1944, 49, 87.

[14] Brandly, C. A., Hanson, R. P., Lewis, S. H., Winslow, N. S., Hoyt, H. H., Pritchard, W. R., and Nerlinger, C. M., *Cornell Vet.*, 1947, 37, 324.

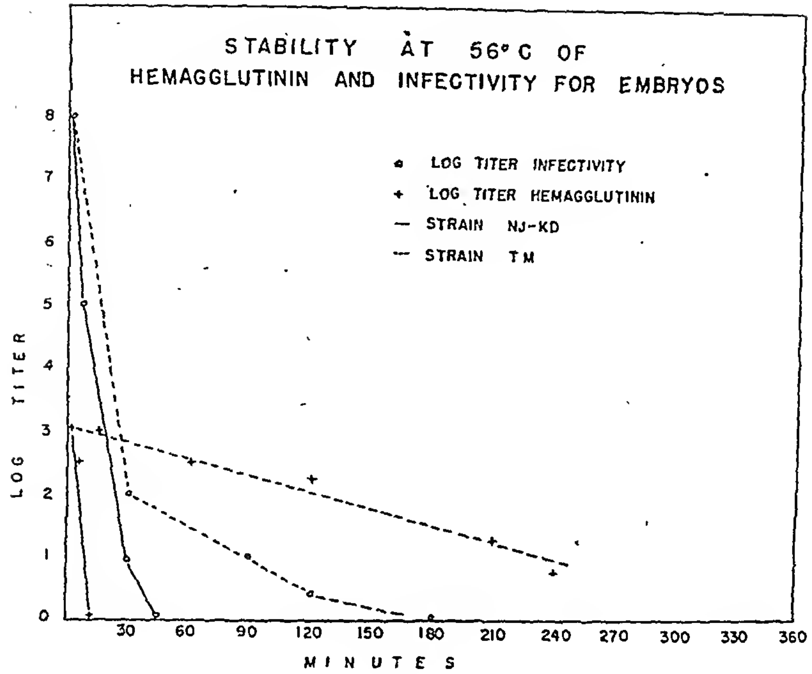

FIG. 1.

The stability of the hemagglutinins is depicted in Table II. Six periods at geometrically increasing intervals, beginning with 15 minutes and ending with 240 minutes, define the stability range of the hemagglutinins of the virus strains observed. The titer of all strains before treatment was approximately 1-1280, which is the usual titer obtained with red blood cells from selected fowls utilized in this study. Three of the 24 strains lost the power to agglutinate red cells within 15 minutes of heat treatment. Of these, FRB and HHT56 had low titers and NJ-KD no titer at all at 10 minutes. At 5 minutes, however, NJ-KD had a significant titer. Six of the 24 strains showed no hemagglutinins at 30 minutes heating. By 60 minutes the number of strains failing to agglutinate erythrocytes increased to 11 and after 2 hours heating to 13. Four hours of heating destroyed the agglutinin of all but 10 of the 24 strains. Four strains, KL, Ber, Cle and M, persisted for 6 hours. No agglutinins have ever been observed after 8 hours of heating at 56°C.

Immediately the question is asked: Is the embryo infectivity (EI) inactivated at the same rate as the hemagglutinin? Fig. 1 presents exploratory data that can be explained only by assuming distinct inactivation rates.

Discussion. Great variation in the stability of the hemagglutinin of individual Newcastle virus strains is apparent from the data given in Table II. Additional differences can be best shown by certain comparisons with studies made on influenza virus strains. Lauffer[5] presented evidence that the site of inactivation of the hemagglutinin and the infectivity for embryos was quite distinct. That Newcastle virus hemagglutinin and infectivity for embryos are inactivated independently is shown in Fig 1. Significant is the fact that demonstrable hemagglutinin of the NJ-KD strain is destroyed before demonstrable infectivity for embryos is lost. The infectivity of the TM strain, on the contrary, is inactivated more rapidly than the hemagglutinin. Most strains having hemagglutinins of low heat stability resemble NJ-KD in their HA/EI stability and most strains having hemagglutinins of high stability resemble TM.[15] Apparently, infectivity of the virus for embryos has a narrower range of heat stability than the virus hemagglutinin. The former ranged from 30 to 180 minutes, as compared to 5 to 360 minutes for the latter. The descending order of susceptibility to heat treatment obtained by the Henles for certain activities of influenza virus is not paralleled with Newcastle virus. With the latter either activity, the HA or the EI, may be destroyed first.

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[15] Upton, E., 1948. Thesis submitted for degree of Master of Science, University of Wisconsin.

The ability of the virus to fix complement in the presence of immune serum is very stable under heat treatment, withstanding 100°C for at least an hour. Using only three strains, 0.01% formalin failed to increase the stability of the hemagglutinin. The possibility still remains that certain other Newcastle virus strains, like some of the influenza strains, may have hemagglutinins more stable to heat in the presence of traces of formalin.

Comparisons of heat resistance of the hemagglutinating activity of the strain with the locality from which it was isolated suggests that a survey, based on many strains, may show a close correlation between them, especially if the time of isolation is considered. All five strains obtained from the eastern seaboard of the United States were of low heat resistance, 4 having either very low or no stability at 15 minutes. All 8 Canadian strains were highly heat resistant, 7 of them having hemagglutinins persisting for 2 to 4 hours. The strains from the Middle West showed more variation in their stability. When the time of isolation of these strains is taken into consideration, it is noted that of 3 obtained in 1946, all were of medium resistance; of 2 obtained in 1947, 1 was of medium and 1 of high resistance; and of 3 obtained in 1948, 1 was of low and 2 were of high resistance. It is possible that a shift in the heat resistance of the midwest strains is occurring and that it might be related to mutation of existing strains or to the introduction of new strains. The existence of such a possibility merits further investigation by this and other methods of strain characterization.

Conclusion. The stability of the hemagglutinin of different Newcastle disease virus strains, when subjected to increased temperatures, has been found to vary over a wide range. The stability of the hemagglutinin of 1 isolate was destroyed at 56°C in a period as short as 5 minutes, and that of another was diminished only after 6 hours exposure. The stability of the hemagglutinin of Newcastle virus is discussed in relationship to influenza viruses. A relation of heat stability of strains to place and time of isolation is observed.

16900

Comparative Nutritive Value of Butter and Vegetable Fats Under Conditions of Low Environmental Temperature.*

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It has been generally accepted by nutritionists that, aside from differences in vitamin potencies, animal and vegetable fats have essentially the same nutritive value. On diets containing an adequate amount of B

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Contribution No. 208 from the Department of Biochemistry, University of Southern California.

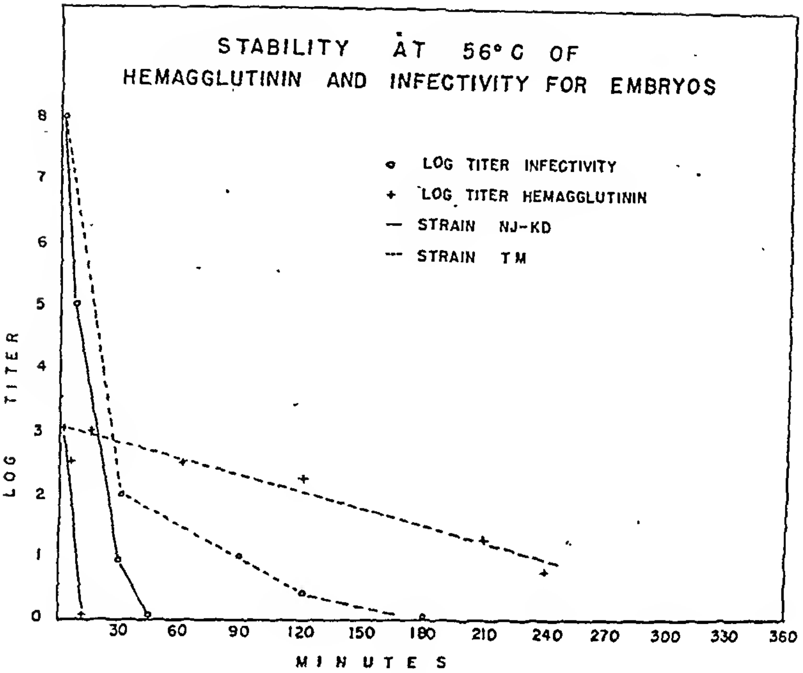

FIG. 1.

The stability of the hemagglutinins is depicted in Table II. Six periods at geometrically increasing intervals, beginning with 15 minutes and ending with 240 minutes, define the stability range of the hemagglutinins of the virus strains observed. The titer of all strains before treatment was approximately 1-1280, which is the usual titer obtained with red blood cells from selected fowls utilized in this study. Three of the 24 strains lost the power to agglutinate red cells within 15 minutes of heat treatment. Of these, FRB and HHT56 had low titers and NJ-KD no titer at all at 10 minutes. At 5 minutes, however, NJ-KD had a significant titer. Six of the 24 strains showed no hemagglutinins at 30 minutes heating. By 60 minutes the number of strains failing to agglutinate erythrocytes increased to 11 and after 2 hours heating to 13. Four hours of heating destroyed the agglutinin of all but 10 of the 24 strains. Four strains, KL, Ber, Cle and M, persisted for 6 hours. No agglutinins have ever been observed after 8 hours of heating at 56°C.

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[15] Upton, E., 1948. Thesis submitted for degree of Master of Science. University of Wisconsin.

The findings are summarized in Table I. Data for the cold room series were computed on the basis of the top 10 animals in each group to minimize variations in averages due to early deaths, infection, and atypical responses on the part of individual rats. Growth was markedly reduced in all rats under cold room conditions. Gain in body weight was most marked on the cottonseed oil diet and least on the ration containing margarine fat. In the room temperature series gain in body weight was most pronounced on the butter fat diet and least for margarine fat. The differences in growth on the various rations, however, either under cold room or room temperature conditions, were not sufficiently marked to be statistically significant.

Data on food consumption and the relative efficiency of the various diets for the building of body tissues are summarized in Table II. The findings indicate that for the first 4 weeks of feeding both under cold room and room temperature conditions virtually no difference in relative efficiency as measured by the ratio of gain in weight × 100 to the calories consumed was obtained for diets containing cottonsed oil, corn oil, or butter fat. Values were somewhat lower in the case of margarine fat, but in view of the small number of animals employed it is questionable whether these differences are significant. Values for the cold room series were less than for the room temperature series, a finding due, at least in part, to the increased metabolism of animals maintained under cold room conditions.

The findings indicate that butter fat, margarine fat, cottonseed oil, and corn oil have substantially the same nutritive value as judged by gain in body weight and efficiency of food utilization of young rats maintained at low environmental temperatures and fed diets differing only in the source of dietary fat.

Summary. Immature female rats were

TABLE II. Summary Table Showing Average Gain in Weight, Average Food Consumption and Ratio of Increase in Weight to Calories Consumed for the First 4 Weeks of Feeding.

Dietary fat	Avg gain in body wt,* g	Food consumption (g/day) on following wks of experiment 1st	2nd	3rd	4th	Avg total food intake per rat for first 4 wks of feeding*† g	Calories	Efficiency‡
		Cold room series (10 animals per group).						
Cottonseed oil	62.4 ± 2.9	11.7	13.2	13.4	14.8	371.7 ± 9.3	1784.2 ± 44.6	3.50 ± .10
Corn oil	62.4 ± 4.3	11.4	13.0	13.0	15.2	368.2 ± 9.7	1767.4 ± 45.0	3.54 ± .18
Margarine fat	58.9 ± 3.4	11.3	13.1	13.6	14.2	365.4 ± 11.0	1753.9 ± 50.0	3.36 ± .05
Butter fat	63.0 ± 3.1	11.3	13.6	14.2	15.1	379.4 ± 10.0	1821.1 ± 49.8	3.46 ± .13
		Room temperature series (6 animals per group).						
Cottonseed oil	86.3 ± 2.6	9.6	10.5	11.3	11.6	301.0 ± 6.9	1444.8 ± 33.0	5.97 ± .19
Corn oil	91.5 ± 5.8	8.9	11.4	11.4	12.3	308.0 ± 14.7	1478.4 ± 70.4	6.19 ± .16
Margarine fat	83.7 ± 2.9	10.0	12.3	13.0	12.8	336.7 ± 11.9	1616.2 ± 57.2	5.18 ± .15
Butter fat	93.3 ± 8.7	9.4	11.0	12.0	10.8	302.4 ± 21.0	1451.5 ± 100.8	6.42 ± .28

* Standard error of the mean; see footnote to Table I.

† The calorie value of these rations was approximately 4.8 calories per gram of diet.

‡ $\frac{\text{G increase in weight}}{\text{Calories consumed}} \times 100$

TABLE I.
Comparative Effects of Butter and Vegetable Fats on the Gain in Body Weight of Immature Rats Maintained Under Cold Room and Room Temperature Conditions.

Dietary fat	No. of rats	Initial body wt., g	Gain in body wt 8 wk period,* g
		Cold room series	
Cottonseed oil	10	65.5	110.0 ± 3.8
Corn oil	10	65.1	100.4 ± 6.4
Margarine fat	10	65.3	94.7 ± 4.2
Butter fat	10	64.4	100.6 ± 4.6
		Room temperature series	
Cottonseed oil	6	64.3	129.7 ± 4.1
Corn oil	6	64.7	138.3 ± 7.9
Margarine fat	6	64.7	119.7 ± 6.5
Butter fat	6	64.2	139.8 ± 9.3

* Standard error of the mean $\sqrt{\frac{\Sigma d^2}{n-1}} / \sqrt{n}$

vitamins no significant difference in growth has been observed under room temperature conditions in immature rats fed diets containing butter fat or various vegetable oils.[1-3] Available data indicate, however, that requirements for essential nutrients may be significantly increased by exposure to cold and other conditions of "stress."[4] It was felt that if differences exist in the nutritive value of fats, these might be accentuated under the stress of low environmental temperature. In the present study immature female rats were raised to maturity on purified rations containing butter and vegetable fats and their rate of growth determined under conditions of low environmental temperature.

Procedure and Results. Four experimental rations were employed in the present experiment, differing only in the source of fat. These consisted of 53.5% sucrose, 22.0% casein,[†] 4.5% salt mixture,[‡] and 20.0% of either cottonseed oil, corn oil, margarine fat or butter fat.[§] To each kg of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 5 mg and choline chloride 1.2 g. Each rat also received once weekly a vitamin A-D concentrate[‖] containing 150 U.S.P. units of vitamin A and 15 U.S.P. units of vitamin D together with 3 mg of alpha-tocopherol acetate. Seventy-two female rats of the Long-Evans strain were selected at 28 to 30 days of age and an average weight of 64.9 g for the present experiment. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces, and were fed the above diets *ad lib.* Feeding was continued for 8 weeks. Experiments were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2±1.5°C, and (2) under standard labora-

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[1] Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *Arch. Biochem.*, 1945, **7**, 143.

[2] Deuel, H. J., Jr., Movitt, E., Hallman, L. F., and Mattson. F., *J. Nutrition*, 1944, **27**, 107.

[3] Deuel, H. J., Jr., Greenberg, S. M., Savage, E., and Fukui, T., unpublished data.

[4] Ershoff, B. H., *Physiol. Rev.*, 1948, **28**, 107.

[†] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[‡] Sure's Salt Mixture No. 1,[5].

[5] Sure, B., *J. Nutrition*, 1941, **22**, 499.

[§] The fats were obtained from the following sources: Cottonseed oil, Wesson Oil, and Snowdrift Sales Co., New Orleans, La., corn oil, Corn Products Refining Co., Argo, Ill.; margarine fat, Best Foods, Inc., New York, N. Y.; butter fat, Knudsen Creamery Co., Los Angeles, Calif. The butter and margarine were melted, and the water and protein separated by centrifugation. The fat was poured off and mixed to give a homogeneous sample.

[‖] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

expected that with higher concentrations of carbon dioxide stronger stimuli would be required to evoke responses.

How these factors combine, and how carbon dioxide affects the action of other convulsants are being studied now.

Summary. Monkeys fitted with bilateral screw-in electrodes and immobilized with dihydro-beta-erythroidine were artificially ventilated with various concentrations of carbon dioxide-oxygen for different periods of time. At the end of these periods they were stimulated with threshold and super-threshold voltages, and it was found that concentrations of carbon dioxide over 20% prevented seizures when inhaled for 3 minutes.

16902

Central Inhibitory Effects of Carbon Dioxide. III. Man.

G. H. POLLOCK, S. N. STEIN, AND K. GYARFAS. (Introduced by W. S. McCulloch.)
From the Department of Psychiatry, University of Illinois, Illinois Neuropsychiatric Institute.

Previous investigations have shown that inhalation of carbon dioxide prevents electrically induced cortical seizures in cats[1] and monkeys.[2] This study was undertaken to note whether this phenomenon was also seen in man.

The subjects chosen were 18 carefully selected patients of varying ages. Fourteen were neurotics and the remaining 4, psychotics.* None had physical disability. The nature of the investigations were explained to them or their relatives prior to their volunteering for the test.

Each patient inhaled a commercially prepared mixture of known carbon dioxide concentration in oxygen for a definite period of time. At the end of this time an electrical stimulus sufficient to cause convulsion was given for 0.5 sec. from on Offner electroshock apparatus. As control, this same electrical stimulus was given after 5 minutes without carbon dioxide. Electroencephalograms were recorded with the Goodwin non-blocking EEG amplifier with ink-writing oscillograph.

The electrical stimulus without carbon dioxide resulted in grand mal seizures. It was found that concentrations of carbon dioxide from 15% to 30% routinely prevented the electrically induced seizures. With 30% carbon dioxide in oxygen, 30 seconds of inhalation sufficed. Slightly longer periods were required with 15 and 20%. With these mixtures of carbon dioxide several cases had decerebrate seizures, which will be fully described by Gyarfas *et al.*[3]

Haldane and Smith[4] found that 10% CO_2 was the upper limit which man could breathe without becoming unconscious. Lennox and Cobb[5] stopped petit mal seizures by having patients breathe high concentrations of CO_2 in air. Both show the central depressant action of CO_2 in man. Heinbecker[6] and Bishop,[7] Necheles and Gerard,[8] Hettwer[9] and raised to maturity under cold room and room temperature conditions on purified rations differing only in the source of fat. The fats employed were cottonseed oil, corn oil, margarine fat, and butter fat. Gain in body weight was significantly reduced in all rats under cold room conditions. No significant difference was observed, however, either under cold room or room temperature conditions, in gain in body weight on the various diets employed.

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[1] Pollock, G. H., Thesis, Univ. of Illinois College of Medicine, 1948.

[2] Stein, S. N., and Pollock, G. H., PROC. SOC. EXP. BIOL. AND MED., in press.

* Thanks are due to the Chicago State Hospital for help with the selection of some of these patients and assistance in contacting relatives and arranging for voluntary transfers to Illinois Neuropsychiatric Institute.

[3] Gyarfas, K., Pollock, G. H., and Stein, S. N., PROC. SOC. EXP. BIOL. AND MED., in press.

[4] Haldane, J., and Smith, J. L., *J. Path. and Bact.*, 1892, **1**, 168.

[5] Lennox, W. G., and Cobb, S., *Medicine*, 1928, **7**, 105.

[6] Heinbecker, P., *Am. J. Physiol.*, 1929, **89**, 58.

[7] Heinbecker, P., and Bishop, G. H., *Am. J. Physiol.*, 1931, **96**, 613.

[8] Necheles, H., and Gerard, R. W., *Am. J. Physiol.*, 1930, **93**, 318.

[9] Hettwer, J. P., *Am. J. Physiol.*, 1938, **122**, 275.

16901

Central Inhibitory Effects of Carbon Dioxide. II. *Macacus rhesus.*

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Pollock's observation that carbon dioxide prevents electrically induced cortical seizures in cats suggested further study on primates.[1] Since the normal electroencephalograph of the *Macacus rhesus* is so well known, and it is not difficult to extrapolate from it to the human, this animal was chosen for the primate study.

Tracheal intubation, exposure of both femoral veins and of the calvarium were performed under ethyl ether anesthesia. Artificial respiration was started after an initial intravenous injection of 20 mg of dihydro-B-erythroidin hydrobromide and was maintained at 45 cc per stroke, 30 strokes per minute. 180 mg of dihydro-B-erythroidin hydrobromide in 300 cc of normal saline was administered as a slow drip throughout the experiment. Bilateral screw-in electrodes were placed 4 mm posterior to the coronal suture and 6 mm lateral to the sagittal suture. These electrodes were connected to a double-pole-double-throw switch allowing alternate connections to the input of a Goodwin non-blocking EEG amplifier with ink-writing oscillograph or to the output of a Lab-Tronics Stimulator (Model 3). The EEG was recorded concurrently with the EKG picked up from the right fore and left hind limbs.

Routinely the animals were ventilated with various concentrations of carbon dioxide in oxygen for intervals of 1 to 9 minutes and, on termination of each period of respiring carbon dioxide, stimulated with threshold and superthreshold voltages. A "saw-tooth" stimulating frequency of 60 impulses per second and a falling phase of 10 sigma were used throughout the experiment. It was found that concentrations of carbon dioxide below 15%, when administered for 3 minutes, would not prevent electrically-induced cortical seizures. When these concentrations were given for periods longer than 3 minutes, suppression of seizures occurred, which was more marked as the concentration of carbon dioxide approached 15%. Concentrations of carbon dioxide over 20% prevented the seizure response. Duration and intensity of the stimuli did not markedly affect the result. It appears that in *Rhesus macacus* the anticonvulsive level of carbon dioxide lies somewhere between 15% and 20%. The changes in the EEG and EKG found with carbon dioxide were similar to those found in cats by Pollock.[1]

Lorente de No' found that the rise of the membrane potential due to carbon dioxide is roughly proportional to the logarithm of its concentration, and this was accompanied not only by a decrease in fatiguability but also by a rise in threshold.[2] It is also known that carbon dioxide alters the pH, oxygen tension and blood flow of the brain.[3] Hence it is to be tered to the patients in sufficient concentration for a sufficient length of time to prevent the convulsant action of the current. The concentration of carbon dioxide varied from 15 to 30% and the duration of administration from 25 to 170 seconds. A description of the technic and of other details has been given by Pollock *et al.*[5]

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[1] Pollock, G. H., Thesis, Univ. of Ill., College of Medicine, 1948.

[2] Lorente de No', R., Studies from the Rockefeller Institute for Medical Research, Vol. 131.

[3] Roseman, E., Goodwin, C. W., and McCulloch, W. S., unpublished data.

Administration of carbon dioxide produced, in all our cases, the following convulsive phenomena: Initial periocular twitching followed by either an extensor hypertonus in all extremities or, more often, slight flexor spasm in the arms and extension in the legs, consecutive transitory plastic tonus, increasing occipital rigidity and opisthotonus, finally, a high degree of extensor rigidity appeared in all limbs, the fingers in *main d'accoucheur* position, toes in flexion. The dilated pupils reacted to light on all but 2 occasions. The tendon and skin reflexes could not be elicited on account of the muscular rigidity. Pathological reflexes were not seen during or after the convulsions though in some cases they preceded them. No tongue biting, incontinence, or postconvulsive stupor appeared. The respiratory and circulatory changes observed were those described by Meduna and Gyarfas.[2] The fits did not appear to be self-limiting. E.E.G., though obscured by muscular movements, was always clearly not of the grand mal type. The electrical activity of the cortex returned to normal shortly after discontinuing inhalation of carbon dioxide.

The clinical symptoms, the E.E.G., and the absence of postseizure stupor, correspond to decerebrate seizures. Our observations indicate, therefore, that the apparently different types of convulsive phenomena described by Meduna and Gyarfas[2] represent only different phases of one activity.

Summary. 1. Combination of E.S.T. with CO_2 inhibits the convulsion.

2. Inhalation of 30% CO_2 and 70% O_2 produced in all cases observed convulsive phenomena of decerebrate character.

16904

Mode of Action of Desoxypyridoxine.

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Antagonisms caused by metabolite analogues are generally accepted as being due to a competition between the analogue and the natural substrate for some enzyme system of the cell.[1] The inhibitor is thought to occupy a space on the surface of an enzyme which would normally be used by the metabolite. The relatively high ratios of analogue to metabolite usually required are considered as a reflection of relative affinity of the enzyme protein for the metabolite and its analogue. While these concepts have served as useful tools and have perhaps been entirely valid in many cases, it has also been apparent that they must be amplified to cover more complex situations; for example, where the substance used is a structural analogue of a vitamin which functions in the form of a coenzyme with a low dissociation constant. Such a case is offered by the action of desoxypyridoxine in competing with the vitamin B_6 group. This compound, 2,4-dimethyl-3 hydroxy-5 hydroxymethylpyridine, was reported by Ott[2] to act as a powerful antagonist of pyridoxine in the chick. Two moles of desoxypyridoxine counteracted 1 mole of pyridoxine when the latter was limiting. Emerson[3] showed that, in the rat, approximately 50 parts of desoxypyridoxine brought on the

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[1] Woolley, D. W., *Advances in Enzymology*, 1946, 6, 129.

[2] Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 125.

[3] Emerson, G. A., *Fed. Proc.*, 1947, 6, 406.

Lorente de No'[10] found that CO_2 raised the threshold of axons of the somatic and the autonomic nervous systems. Lorente de No'[10] showed that the carbon dioxide raised the membrane potential and lengthened the refractory period of nerve. The central inhibitory effects of the carbon dioxide may well be due to the increase of membrane potential and threshold. Moreover, breathing carbon dioxide increases cerebral blood flow by dilating cerebral arterioles, increases respiratory rate and so the oxygenation of blood, shifts the hemoglobin dissociation curve so that the blood releases more oxygen to the tissues and shifts cortical pH to the acid side. In the explanation of the central inhibitory action of the carbon dioxide these factors must all be considered.

Summary. Eighteen patients were ventilated with various concentrations of carbon dioxide-oxygen for varying lengths of time and then were stimulated with super-threshold shocking current. Concentrations of carbon dioxide from 15-30% routinely prevented electrically induced seizures; with 30% carbon dioxide, 30 seconds of inhalation sufficed; with 15-20% mixtures, slightly longer periods of time were required.

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[10] Lorente de No', Raphael, Studies of the Rockefeller Institute, vol. 131, 1937, New York.

16903

Central Inhibitory Effects of Carbon Dioxide. IV. Convulsive Phenomena.

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Convulsive phenomena produced by inhalation of carbon dioxide were first mentioned by Waters.[1] Meduna and Gyarfas[2] observed 3 types of convulsive manifestations. The first type is the socalled adversive seizure which consists of conjugate deviation, extension of the arm and flexion of the leg on the side toward which the eyes turn. These fits are not accompanied by any change in the peripheral reflexes and can be explained as tonic neck reflexes due to premotor release.

The second type consists of flexion in the upper with extension in the lower extremities and is accompanied by slight rhythmical twitching. No changes in the peripheral reflexes accompany this seizure. The third type consists of sudden dilation of the pupils, absence of any reaction to light, opisthotonus, extension of all extremities, and flexion of the toes. This type of seizue was interpreted by Meduna and Gyarfas as a symptom of decortication.

Pollock,[3] Stein and Pollock,[4] Pollock *et al.*[5] demonstrated that carbon dioxide in certain concentrations inhibits the action of cortical convulsants.

Pollock and Bain[6] showed that changes in the metabolism of the brain produced by inhalation of carbon dioxide differ from those produced by cortical convulsants. In order to establish the anticonvulsant effect of carbon dioxide in man 92 experiments were carried out in 18 patients in the following way: First, the electro-shock dose of every patient was established, then carbon dioxide was adminischlorides. Pyridoxal phosphate was standardized against a relatively pure sample[10] and was used as the calcium salt. Desoxypyridoxine phosphate will be described later. Adenosine triphosphate (ATP) was used as the sodium salt. Conventional Warburg technics were employed throughout.

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[1] Waters, R. M., *New Orleans Med. and Surg. J.*, 1937, **219**, 90.

[2] Meduna, L. J., and Gyarfas, K., Diseases of the Nervous System, in press.

[3] Pollock, G. H., Thesis, Univ. of Illinois, College of Medicine, 1948.

[4] Stein, S. N., and Pollock, G. H., PROC. SOC. EXP. BIOL. AND MED., in press.

[5] Pollock, G. H., Stein, S. N., and Gyarfas, K., PROC. SOC. EXP. BIOL. AND MED., in press.

[6] Pollock, G. H., and Bain, J. A., manuscript in preparation.

Results. As outlined above, several possible sites of action of desoxypyridoxine are evident. These may be divided into two categories: (1) the inhibitions possible if the active agent is desoxypyridoxine, (2) the inhibitions possible if the active agent is desoxypyridoxine phosphate. While the second category proved to be correct, as will be shown later, it was still necessary to prove that there does not also exist an effect of desoxypyridoxine itself. This proof is provided in the first section. The tyrosine decarboxylase system employed did not include reactions A (Fig. 1) hence any effect of desoxypyridoxine (or its phosphate) on the conversion of pyridoxine or pyridoxamine to pyridoxal phophate could not be studied in this system.

Action of desoxypyridoxine. Since the results show that desoxypyridoxine as such has no effect upon the systems shown in Fig. 1, only a brief description of the experiments will be given. It was first necessary to test the possibility that desoxypyridoxine interfered with tyrosine decarboxylase presumably by displacing the pyridoxal phosphate. Beiler and Martin[11] have already reported that desoxypyridoxine was ineffective in this system up to concentrations as high as 300 γ per ml. In our hands concentrations as high as three times this level had no effect. Further, such concentrations had no effect upon the aspartic-glutamic transaminase from heart muscle[12,13] or upon the tryptophanase system of *E. coli*[14] both of which have somewhat higher dissociation constants than tyrosine decarboxylase and hence might be expected to show some degree of competition. Once pyridoxal phosphate is associated with the tyrosine decarboxylase protein, desoxypyridoxine does not displace it. Yet if the latter were allowed to reach the apoenzyme first it might prevent the combination of pyridoxal phosphate and apoenzyme. For this purpose a preparation was employed which at saturation had a Q_{CO_2} of 676. It responded in a linear manner to pyridoxal phosphate up to 8 mγ (calculated as the free acid) at which point the Q_{CO_2} was 460. It was reasoned that the ideal conditions for demonstrating any effect of desoxypyridoxine upon the combination of apoenzyme and coenzyme would be under conditions where the coenzyme was decidedly limiting since any competition would be immediately evident in a decreased rate of tyrosine decarboxylation. A point was therefore chosen approximately halfway up the region of linear response (3.8 mγ free acid) with the result (Table I) that desoxypyridoxine had no effect upon the combination of coenzyme and apoenzyme in spite of the fact that it was given adequate access to the enzyme before the pyridoxal phosphate was supplied. Similar results were obtained with a purified horse-heart transaminase preparation carried through the second precipitation in the procedure of O'Kane and Gunsalus[13] at which point it was 85% resolved.

Since from these data, desoxypyridoxine does not act on tyrosine decarboxylase (C, Fig. 1) the possibility remained that it acted by competing with pyridoxal for conversion to pyridoxal phosphate (point B, Fig. 1). It was reasoned that if ATP were made the limiting factor in the conversion of pyridoxal to pyridoxal phosphate, effects of replacing pyridoxal by desoxypyridoxine on the surface of the converting enzyme would be immediately evident. In this type of experiment a preparation was employed which had a very active converting system. Not very much is known about this enzyme and its occurrence in a given preparation is not exactly pre-signs of B_6 deficiency when a purified diet supplemented with pyridoxine was employed and that this ratio changed to 175 parts when a natural diet was fed. Additional studies by Mushett *et al.*,[4] Porter *et al.*[5] have demonstrated that desoxypyridoxine produces the pathological and biochemical symptoms associated with a vitamin B_6 deficiency so that there is no question that desoxypyridoxine antagonizes vitamin B_6. These studies revealed, however, that the antagonism was evident only when the vitamin B_6 supply was sub-optimal. With adequate vitamin B_6, desoxypyridoxine had little effect even at relatively high ratios. These conclusions are difficult to explain on a metabolite-analogue competition basis so that it was early realized that a more complex situation was present. Direct enzymatic studies were therefore undertaken to attempt to determine the mechanism of inhibition by desoxypyridoxine. The enzyme employed was tyrosine decarboxylase for which pyridoxal phosphate, a member of the vitamin B_6 group, is the coenzyme.[6] It was assumed that these studies would bear some relationship to the mode of action of desoxypyridoxine in the animal body but the concepts developed experimentally here may require some modification when applied to the complex situation in the animal.

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[10] Gunsalus, I. C., Umbreit, W. W., Bellamy, W. D., and Foust, C. E., *J. Biol. Chem.*, 1945, **161**, 743.

[11] Beiler, J. M., and Martin, G. J., *J. Biol. Chem.*, 1947, **169**, 345.

[12] Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, 1945, **161**, 559.

[13] O'Kane, D. E., and Gunsalus, I. C., *J. Biol. Chem.*, 1947, **170**, 425.

[14] Wood, W. A., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1947, **170**, 313.

The substrate competition concept is based upon the early studies of Quastel and Wooldridge[7] upon malonate inhibition of succinate transformations and this concept has largely colored the thinking of subsequent observers. However, when one is dealing with a vitamin analogue, the points of competition may be multiplied and a simple concept may require modification. The case of vitamin B_6 may be diagrammed as in Fig. 1. The known coenzyme form of the vitamin B_6 group is pyridoxal phosphate, yet in the diet pyridoxine, pyridoxal, and pyridoxamine are, within limits of stability and adsorption, essentially equally effective and have been shown to be converted into pyridoxal phosphate.[8] Desoxypyridoxine might act at points A, B, or C or it might be converted into desoxypyridoxine phosphate which could compete by uniting with the apo-tyrosine decarboxylase, or by displacing pyridoxal phosphate from the holoenzyme. Since the analogue could act at one or at more than one of these points, it hardly seemed likely that a simple concept of substrate competition would suffice to describe the action of desoxypyridoxine. It therefore appeared worthwhile to attempt to distinguish experimentally between the possibilities mentioned particularly since enzymatic studies involving the possible interactions outlined above have been somewhat lacking.

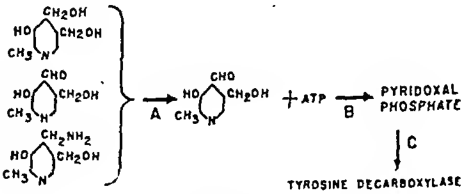

FIG. 1.
Reactions of members of the vitamin B_6 group.

Methods and materials. *Streptococcus faecalis*, strain R, was used as a source of tyrosine decarboxylase. For the preparation of the active (holo) enzyme the organism was grown at 25°C, 16-18 hours, in a medium composed of 1% each of tryptone, yeast extract, and glucose with 0.5% K_2HPO_4. The cells were harvested by centrifugation, washed with distilled water, and the vacuum dried preparation used as a source of the enzyme. The apoenzyme was prepared similarly using a medium deficient in the vitamin B_6 group.[9] The decarboxylation of tyrosine was measured at 37°C in 0.06 M acetate buffer at pH 5.5 as previously described.[6] Pyridoxal and desoxypyridoxine were used as their hydro-

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[4] Mushett, C. W., Stebbins, R. B., and Barton, M. N., *Trans. N. Y. Acad. of Sciences*, 1947, **9**, 291.

[5] Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, 1947, **167**, 573.

[6] Umbreit, W. W., Bellamy, W. D., and Gunsalus, I. C., *Arch. Biochem.*, 1945, **7**, 185.

[7] Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, 1927, **21**, 1224.

[8] Bellamy, W. D., Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, 1945, **160**, 461.

[9] Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, 1945, **50**, 95.

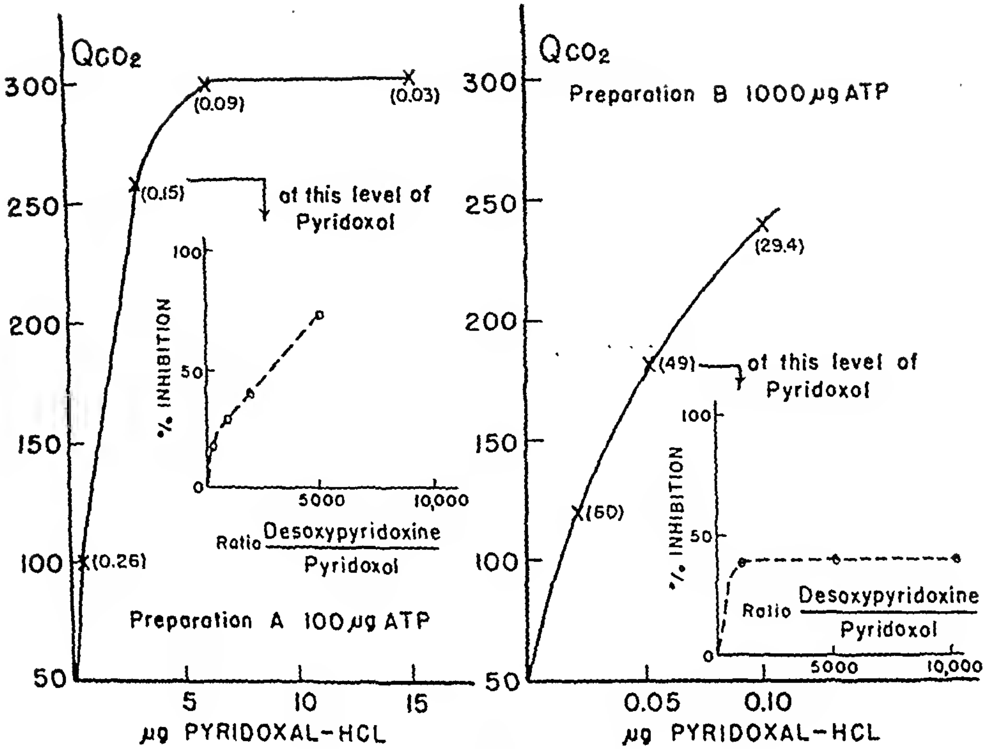

FIG. 2.

Enzymatic conversion of desoxypyridoxine to its phosphate and its interference with tyrosine decarboxylation.

In each case, 1 mg of the enzyme preparation was incubated with ATP and desoxypyridoxine for 15 min., pyridoxal added, in amounts indicated, incubated for 10 min., then tyrosine added and the rate of tyrosine decarboxylation determined. The solid lines show the rate of tyrosine decarboxylation at various levels of pyridoxal supplied in the absence of desoxypyridoxine and *serve to show that* pyridoxal is the limiting factor and that ATP is in excess. The values in parentheses give the percent of conversion of pyridoxal to pyridoxal phosphate. On the inset graphs the dashed curves show the extent of inhibition by various increments of desoxypyridoxine at the level of pyridoxal indicated.

pyridoxine could not compete to any detectable extent with pyridoxal. However, if pyridoxal were limiting so that some portion of the enzyme were free from pyridoxal, while ATP was at saturation or above, desoxypyridoxine might then become attached to the enzyme and be phosphorylated, the latter form interfering somehow with reaction C which would be inhibited. Data showing that such an inhibition does occur are given in Fig. 2.

Two types of preparations were used, one (preparation A) in which the conversion of pyridoxal to pyridoxal phosphate was relatively weak (0.2-0.3%) and another (preparation B) in which the conversion was relatively good (50-60%). The figures in parentheses on the graphs give the per cent of the pyridoxal supplied which appeared as pyridoxal phosphate (after a 10 minute incubation period). The pyridoxal phosphate was determined by comparing the activity found with that given by purified pyridoxal phosphate with the same preparation. As previously mentioned the conversion of pyridoxal to the phosphate by ATP is not a quantitative reaction and such preparations respond to pyridoxal phosphate in much lower quantities than to pyridoxal and ATP. For example, in the original enzyme preparation described[6] pyridoxal phosphate was 18-21 times as active as pyridoxal and ATP[10] indicating a conversion of approximately 5%. Different preparations of the dried cells of this organism

TABLE I.
Lack of Effect of Desoxypyridoxine on Combination of Pyridoxal Phosphate and Apo-tyrosine Decarboxylase.

Pyridoxal phosphate, mγ	Ratio $\frac{\text{Desoxypyridoxine}}{\text{Pyridoxal phosphate}}$	Rate of tyrosine decarboxylation, Q_{CO_2}
0	0	61.6
1.92	0	158.8
3.84	0	242
3.84	3000	240
3.84	6000	246
7.68	0	448
11.52	0	540
15.36	0	576
19.20	0	676

Desoxypyridoxine, when supplied, incubated with apoenzyme 30 minutes before pyridoxal phosphate supplied.

TABLE II.
Effect of Desoxypyridoxine on the Synthesis of Pyridoxal Phosphate from Pyridoxal and ATP.

γ ATP*	Ratio $\frac{\text{Desoxypyridoxine}}{\text{Pyridoxal}}$	Q_{CO_2} on tyrosine
10	0	160
10	500	173
10	10,000	162
50	0	360
50	500	368
50	10,000	390
100	0	448
100	500	448
100	10,000	444

* $Na_4(ATP) \cdot 3H_2O$.

dictable. We have had preparations capable of converting 50% of the pyridoxal supplied to pyridoxal phosphate while others capable of converting only 1 to 2% or even less have been obtained under apparently the same conditions of cultivation and treatment of the organisms. However, in this case, a preparation actively converting 50% of the pyridoxal supplied was employed. ATP was made the limiting factor and two pyridoxal levels, one low (0.05 γ) and another relatively high (1 γ) were employed. The desoxypyridoxine was incubated with the enzyme for 30 minutes, to allow access to the enzyme, the pyridoxal and ATP were added simultaneously, incubated for 10 minutes to allow reaction B to proceed and the rate of tyrosine decarboxylation measured. The data for the lower level of pyridoxal are given in Table II; since the results with the higher level were similar they have been omitted. Within the limits of error of measurement there is no effect of desoxypyridoxine upon the formation of pyridoxal phosphate from ATP and pyridoxal. This is somewhat surprising since the competition theory as it is usually applied would predict that this reaction would be the site of action of desoxypyridoxine.

Action of desoxypyridoxine phosphate. The previous section has shown that desoxypyridoxine does not influence either the conversion of pyridoxal to its phosphate or the activity of the phosphate once formed. There remained, therefore, the possibility that desoxypyridoxine was not the active agent in the competition but that it was itself converted into desoxypyridoxine phosphate which was the competitive agent. Evidence bearing on this point was first sought in the conversion system (B, Fig. 1). The previous section has shown that when ATP was the limiting factor and adequate pyridoxal was available, desoxypyridoxal phosphate. The individual treatments are plotted as discrete points on the curve. In one case desoxypyridoxine phosphate and pyridoxal phosphate were mixed and added to the preparation simultaneously on the assumption that this could provide an equal chance for both to reach the enzyme. They were then incubated for various periods up to 30 minutes before tyrosine decarboxylation was measured. In another treatment desoxypyridoxine phosphate was supplied first, incubated with the apoenzyme for 30 minutes, pyridoxal phosphate supplied, equilibrated for 10 minutes and tyrosine decarboxylation measured. Within the errors of measurement all of these combinations where desoxypyridoxine phosphate had an equal or better chance to reach the apoenzyme than pyridoxal phosphate, essentially the same degree of inhibition is observed. If, however, as shown in curve B, pyridoxal phosphate is supplied to the apoenzyme first and the combination between the two permitted, desoxypyridoxine phosphate supplied later has much less effect; in fact, the total inhibitions reached at the highest ratio was only 12%.

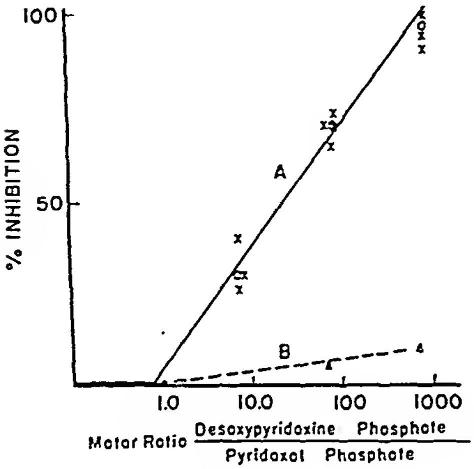

FIG. 3.

Competition between desoxypyridoxine phosphate and pyridoxal phosphate for tyrosine decarboxylase apoenzyme.

In curve A, desoxypyridoxine phosphate incubated with apoenzyme for 30 min. before pyridoxal phosphate added, or desoxypyridoxine phosphate and pyridoxal phosphate added simultaneously. After 10 min. of contact with pyridoxal phosphate, tyrosine was added and the rate of tyrosine decarboxylation determined. In curve B, the pyridoxal phosphate was added before the desoxypyridoxine phosphate. Pyridoxal phosphate used at level of 20 mγ (barium salt) per 3 ml, which is close to the top of the linear portion of the response curve of this preparation.

These data constitute evidence that the inhibition of tyrosine decarboxylase is due to the competition between desoxypyridoxine phosphate and pyridoxal phosphate for the apoenzyme. Pyridoxal phosphate has a greater affinity for this enzyme than desoxypyridoxine phosphate and if it is permitted to combine with the apoenzyme before desoxypyridoxine phosphate is present, the latter has but little effect.

A moment's consideration of the data presented will show that this locus of action of desoxypyridoxine is capable of explaining the results observed with desoxypyridoxine in growth studies. It is only when vitamin B_6 is deficient that there is any great opportunity for either the phosphorylation of desoxypyridoxine or its combination with apoenzymes and thus it is only under these circumstances that a marked inhibitory effect is observed. It is also evident that when one is dealing with a vitamin analogue the simple concept of substrate competition must be modified to include the other possibilities of competition. Further, there is no assurance that other analogues of vitamin B_6 might not act at different loci, hence any general viewpoint of metabolite analogue competition must be amplified. With such amplification it would seem reasonable that many of the present unexplainable phenomena of metabolite analogue competition might become understandable.

Summary. Upon the basis of the data presented, it is concluded that desoxypyridoxine exerts its inhibiting effect by being first converted into desoxypyridoxine phosphate which then competes with pyridoxal phosphate for the apoenzyme. This conclusion offers an explanation for the observation that in the animal, desoxypyridoxine exerts its antagonistic effect primarily under conditions of restricted vitamin B_6 intake.

require different quantities of pyridoxal and ATP to reach either the maximum activity or the activity corresponding to a given level of pyridoxal phosphate. The cause of this difference is not known but it might possibly be related to different degrees of permeability of the cell to pyridoxal (thus preparation A might require 2 γ pyridoxal-hydrochloride to reach an internal concentration equivalent to that which preparation B was capable of reaching with only 0.06 γ). There are some indications that this is indeed the explanation but proof is lacking. However, by the use of two preparations of widely different "converting abilities" it was hoped to avoid complications arising from this effect.

The level of pyridoxal was chosen which gave good activity but which was below the saturation level. Desoxypyridoxine did, under these circumstances, show inhibitions as shown on the inserted graphs in each case. However, relatively high levels of desoxypyridoxine were required. Where the conversion to pyridoxal phosphate is relatively weak, desoxypyridoxine exerts its greatest effect. These data provide presumptive evidence that when ATP is in excess and pyridoxal limiting, there is some degree of inhibition by desoxypyridoxine. Since various other possibilities have been eliminated in the first section, the remaining possibility is that desoxypyridoxine is converted into desoxypyridoxine phosphate and that the phosphorylated derivative is the actual inhibitor.

Unfortunately desoxypyridoxine phosphate is not available nor is its preparation a matter of simple chemistry. The synthesis used by Beiler and Martin[11] is not a good one. Yields of pyridoxal phosphate using pyridoxal as the starting material are rarely more than 0.5% and there is no reason to expect any better yields with desoxypyridoxine. Somewhat improved syntheses have been obtained from pyridoxal by modifications of the original procedure[6] but rarely is more than a 5% conversion obtained. In the case of pyridoxal phosphate an assay method[6] is available which permits one to follow purification of this substance, but with desoxypyridoxine there is as yet no method of specifically estimating the phosphorylated form in a mixture of phosphorylated compounds. As a source of desoxypyridoxine phosphate we therefore employed a preparation* made by treating desoxypyridoxine with phosphoryl chloride, neutralizing the resulting mixture and separating the desoxypyridoxine phosphate as the calcium salt by alcohol precipitations. Such a material is not pure and may contain other substances. However, its organic phosphate content (4.52%) was assumed to be due to desoxypyridoxine phosphate and the amount added was calculated on this basis.

From the data cited previously, desoxypyridoxine phosphate interferes with reaction C (Fig. 1) in some manner. There are two parts to this reaction, one, a combination of the apoenzyme and pyridoxal phosphate, and two, the decarboxylation of tyrosine. The desoxypyridoxine phosphate does not measurably prevent the activity of tyrosine decarboxylase once the coenzyme is attached.

A vacuum dried preparation of *S. faecalis R* grown on a yeast extract medium had a Q_{CO_2} on tyrosine of 393. When 100,000 times as much desoxypyridoxine phosphate was supplied as the pyridoxal phosphate contained in the preparation a Q_{CO_2} of 362 was observed (8% inhibition); with 1 million times as much desoxypyridoxine phosphate the Q_{CO_2} was 372 (5% inhibition). These inhibitions are not significant. Beiler and Martin[11] have reported some degree of inhibition (approximately 30%) of tyrosine decarboxylase with a preparation of desoxypyridoxine phosphate. This type of inhibition has not occurred in our experiments.

Desoxypyridoxine phosphate does, however, interfere markedly with the combination of pyridoxal phosphate and apoenzyme. Data on this point are shown in Fig. 3 in which are plotted the results of a variety of conditions employed in determining the inhibitions at various ratios of desoxypyridoxine phosphate to pyridoxal phosphate. Curve A is the average of several types of experiments designed so that the desoxypyridoxine phosphate would have an equal or better chance of reaching the apoenzyme surface than the

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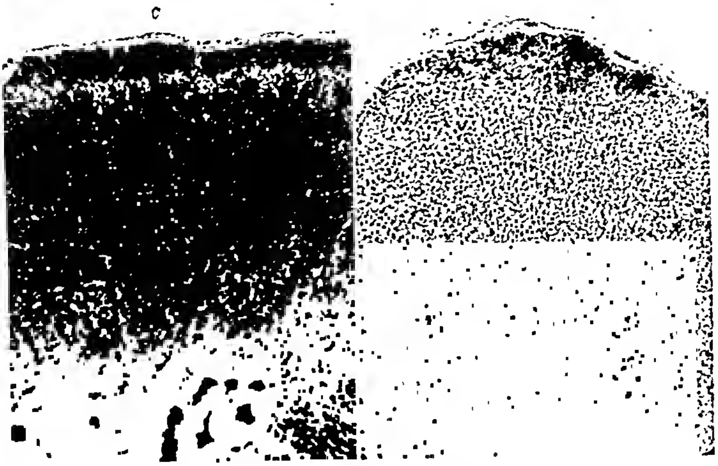
* We are indebted to the Research Laboratories of Merck & Co., Inc., for this preparation.



Frozen sections stained with Sudan III. Photographed with a green Wratten filter No. 29 at 135 diameters magnification and reduced to 68 by the engraver.

FIG. 1. Cortex of the adrenal gland of a normal animal.

FIG. 2. Cortex of the adrenal gland of an animal injected with 1 mg of sodium cyanide followed by ½ mg every half hour for 28 hours. Note depletion of lipids especially in the inner zones while the outer zona glomerulosa is relatively resistant.

bolism. Alterations of carbohydrate metabolism may be prevented by ablation of either the pituitary or adrenal (Evans[5]). The zona glomerulosa, which reacts last to anoxia, is not under control of the pituitary and, at least in the rat, regulates electrolyte balance (Nichols[6]; Deane *et al.*[7]).

The factor(s) responsible for pituitary stimulation in anoxia are uncertain. In anoxic anoxia, the most likely factors are the reduced oxygen tension of the arterial blood and the hypocapnia incident to hyperventilation. That hypocapnia may be a significant factor is indicated by the observation of Hailman[8] and that of Langley *et al.*[9] that the addition of sufficient carbon dioxide to give a partial pressure of 30 to 48 mm Hg prevents the usual anoxic changes in the adrenal gland and also prevents the increased deposition of liver glycogen. The changes in the adrenal are probably the result of the lowered blood carbon dioxide tension *per se*, rather than the resultant disturbance in acid base equilibrium. In unpublished experiments in this laboratory it was found that alkalosis and acidosis induced by oral administration of sodium bicarbonate and ammonium chloride respectively had no effect on adrenal changes in anoxic anoxia.

In this study we have not eliminated the hyperventilation possibility, since the animals did hyperventilate after each injection. In order to avoid this it would be necessary to denervate the carotid body. However, our findings indicate that histotoxic anoxia induced by sodium cyanide produces the same changes in the adrenal as does anoxic anoxia and that these changes can occur while the arterial blood is saturated with oxygen.

Summary. Sodium cyanide was administered in small repeated sublethal doses to rats. The resulting changes in the adrenal gland were hypertrophy and marked depletion of lipids in the inner zones of the cortex. This effect is identical with that of anoxic anoxia and occurs while the arterial oxygen tension is normal.

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[5] Evans, G., *Am. J. Physiol.*, 1935, **114**, 297.

[6] Nichols, J., *Arch. Path.*, 1948, **45**, 717.

[7] Deane, H. W., Shaw, J. H., and Greep, R. O., *Endocrinology*, 1948, **43**, 133.

[8] Hailman, H. F., *Endocrinology*, 1944, **34**, 187.

[9] Langley, L. L., Nims, L. F., Harvey, T. S., and Clarke, R. W., *N.R.C. Committee on Aviation Medicine* Report No. 108, 1943.

16905

Effects of Cyanide Anoxia on Adrenal Gland of the Rat.

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Armstrong and Heim[1] reported that anoxic anoxia induces hypertrophy of the adrenal gland in rabbits. Since that time many papers have appeared on this subject (for references see Tepperman[2] and Nichols[3]). The most characteristic findings are hypertrophy of the gland and depletion of lipids from the cortex. To our knowledge the effects on the adrenal gland of other types of anoxia have not been studied, with the single exception of the work of Messerle[4] who made incidental observations during chronic exposure of pigeons to hydrogen cyanide gas. He reported gross hypertrophy of the gland but on histological observation found no cytological changes. It was thought that additional information on the mechanism of anoxic stimulation of the adrenal gland might be derived from experiments in which the oxygen tension of the arterial blood is normal.

Materials and methods. Twenty male rats of the Long-Evans strain, each weighing 150 g, were used in this study. Preliminary experiments indicated that 2 mg of sodium cyanide would kill a 150 g rat in about 30 minutes; accordingly, a maximum dose of 1 mg of cyanide was adopted for use in the remainder of the experiments. This was sufficient to give a severe but safe level of anoxia. Seven rats were injected subcutaneously with 1 mg of sodium cyanide contained in 1 ml of saline, followed by ½ mg of sodium cyanide every hour for the next 20 hours. Seven animals were injected with 1 mg of sodium cyanide followed by ½ mg of cyanide every half hour for the next 28 hours. The remaining 6 animals were treated as controls, being injected with 1 ml of saline every hour for 20 hours. All animals were killed by a blow on the head and the adrenals removed and placed in 10% formaldehyde for 24 hours. The surrounding fat was then carefully dissected away and the glands weighed. They were cut in half, embedded in gelatin and sectioned on the freezing microtome at 14 microns, stained and mounted as previously described.[3]

Results. The average weights of the adrenal glands were: controls 40 mg, treated 20 hours 45 mg, and treated 28 hours 54 mg. Histologically, the adrenals of the control animals were entirely normal as compared with other normal glands studied in this laboratory (Fig. 1). The glands of the animals treated for 20 hours showed marked depletion of lipids in the inner cortical zones (fasciculata and reticularis), while the zona glomerulosa remained relatively unchanged. The animals treated for 28 hours showed the same adrenal changes except to a more striking degree (Fig. 2); in certain of these animals the lipids were depleted in the zona glomerulosa to almost the same extent as in the inner zones. These histological changes are the same as those that occur in anoxic anoxia.

Discussion. As previously mentioned Messerle[4] reported gross hypertrophy of the adrenal, but no cytological changes, on chronic exposure of pigeons to hydrogen cyanide; however, he used standard hematoxylin and eosin preparations which had been dehydrated in alcohols that would dissolve out the fats.

Adrenal cortical lipid depletion in anoxia is believed to be mediated by way of the pituitary gland since the zones first involved (*i.e.*, *fasciculata* and *reticularis*) are known to be under control of the pituitary; these zones elaborate the fraction of the cortical hormone which influences carbohydrate meta-

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[1] Armstrong, H. G., and Heim, J. W., *J. Aviation Med.*, 1938, **85**, 162.

[2] Tepperman, J., Tepperman, H. M., Patton, B. W., and Nims, L. F., *Endocrinology*, 1947, **41**, 356.

[3] Nichols, J., *J. Aviation Med.*, 1948, **19**, 171.

[4] Messerle, N., *Virchow's Arch.*, 1926, **262**, 305.

TABLE I.
Composition of the Tocopherol-deficient Diet Used.

Basal mixture	%	Supplements to basal mixture	mg/kg
Lard	10	Thiamine chloride	2
Casein (Borden's crude) No. 453	30	Riboflavin	4
Cerelose	54	Pyridoxine	4
Celluration	2	Nicotinic acid	100
Salt mixture (Hawk Oser)	4	Choline	1000
		Vit. K	4
		Para-amino-benzoic acid	300
		Ca pantothenate	10
		Percomorphum oil (ml/kg)	0.2

tribution of acidfast pigment.

Experiments and observations. 1. Uterine response to estrogen. The uterine weights of deficient and non-deficient ovariectomized animals after the administration of alpha-estradiol benzoate are given in Fig. 1. In the age group of 40-50 days, 1 μg of hormone dissolved in 0.2 cc of sesame oil was injected subcutaneously 48 hours before sacrifice. In the groups 120-137 and 280-309 days of age, a total of 4 μg of the estrogen, divided in successive daily doses of 1 μg in 0.2 cc of oil, was injected. The final dose was given 48 hours before sacrifice. Several of the deficient and tocopherol-supplemented animals in the 120 day group had received a total of 11 μg of estradiol benzoate between the 43rd and 78th day. Since their uterine response was similar to that of the other animals in this age group, they have been included in the final observations.

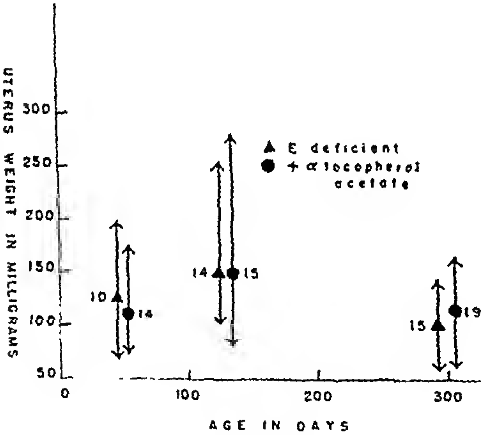

FIG. 1.

Effect of alpha estradiol benzoate upon the uterine weight of spayed rats on a vitamin E-deficient and a tocopherol-supplemented purified diet. The triangles and circles indicate average uterine weights; the figures adjacent to the triangles and circles indicate the number of animals used in the respective series.

There is no significant difference in the uterine response to estrogen between the tocopherol deficient and tocopherol-supplemented animals in the same age group. There is, however, a difference in the response between animals in the 120-137 day and the 280-309 day groups, irrespective to tocopherol administration. Statistical evaluation of the mean uterine weights of these two groups yielded a probability factor of 3.7 indicating that the response of the 4-month-old rat to estrogen is significantly greater than that of the 9-10-month-old animal.

2. Uterine pigmentation. As reported by previous investigators, a brownish pigmentation of the uterus was macroscopically discernible in the non-spayed rats maintained on the tocopherol-deficient diet for approximately 300 days. In sharp contrast, there was no evidence of pigment deposition in the majority of the spayed animals fed the same diet for the same period of time. As would be expected, no pigmentation occurred in either spayed or non-spayed animals receiving the tocopherol-supplemented diet.

Histological examination of the uteri of 8 unspayed deficient animals revealed that the muscle cells of the myometrium were filled with numerous uniformly small acidfast pigment granules. In addition, a considerable number of pigment-containing cells were scattered throughout the intermuscular connective tissue and, to a lesser extent, the endometrial stroma. These cells, presumably macrophages, varied in size and shape, but

16906

Response and Pigmentation of the Uterus in Vit. E-Deficient Rat.*

H. Kaunitz, C. A. Slanetz, and W. B. Atkinson.
(With the technical assistance of R. E. Johnson and A. Fuhr.)
From the Departments of Pathology, Animal Care, and Anatomy, College of Physicians and Surgeons, Columbia University, New York City.

Several investigations have been made concerning the possibility that a relationship exists between the biological action of estrogen and vitamin E.[1-3] Although some of these experiments have suggested an "activating" effect of tocopherol on estrogen, the data have not been conclusive. A related problem, that of the role of the ovaries in the development of the characteristic pigmentation of the uterus during vitamin E deficiency, also remains unsettled. Therefore the present experiments were designed to determine the effect of alpha-tocopherol deficiency on the uterine response to estrogen and the effect of ovariectomy on the development of uterine pigmentation in vitamin E-deficient rats.

Materials. The animals used in the present experiments were obtained in the following manner. Female rats of the "Sherman" strain were reared from weaning on Rockland rat diet. Upon reaching 2-3 months of age they were placed on a vitamin E-deficient diet (Table I) which limited the daily intake of alpha tocopherol to approximately 30 μg per rat.† Within one week after they had been placed on the deficient diet, the animals were mated with non-deficient males. The tocopherol-deficient offspring were used in the present experiments as indicated below. After weaning of the young, the mothers were returned to the Rockland diet for 3 weeks and the same breeding procedure was then repeated.

Within 3 days after birth the young were pooled and 6 females were given to each of several mothers. These young were thereafter considered as "litter-mates". Between 3 and 4 weeks of age they were weaned and ovariectomized. Thirty-nine animals were continued on the vitamin E-deficient diet and 48 were placed on the same ration supplemented with 3 mg of synthetic dl alpha-tocopherol acetate per 100 g diet.‡ Eight unspayed animals were kept on the deficient diet as controls.

The uterine response to estrogen was determined with crystalline alpha-estradiol benzoate as indicated below.§ The hormone was dissolved in sesame oil which was freed of tocopherol by previous treatment with ferric chloride.

For histological examination the uteri were fixed in Bouin's fluid within 10 minutes after the animals were killed. Tissue specimens were dehydrated in ethyl alcohol, embedded in paraffin and sections were cut 7 microns in thickness. Parallel sections were stained with hematoxylin and eosin and with carbol-fuchsin to determine the presence and dis-

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* Aided by a grant from the Williams-Waterman Fund of the Research Corporation.

[1] Spoto, Pompeo, *Z. Vitaminfschg.*, 1940, **10**, 235.

[2] Beerstecher, E., Jr., *Endocrinology*, 1941, **28**, 344.

[3] Tobin, Charles E., and Birnbaum, Jean P., *Arch. Pathol.*, 1947, **44**, 269.

† About one-half of the determinations of the tocopherol content of the basic diet were carried out according to the method of Kaunitz and Beaver.[6] For the remaining tests we are greatly indebted to Drs. Philip L. Harris and Mary L. Quaife of the Distillation Products, Inc.

[6] Kaunitz, H., and Beaver, J. J., *J. Biol. Chem.*, 1944, **156**, 653.

‡ The alpha tocopherol acetate and the other synthetic vitamins were supplied through the courtesy of Dr. Leo A. Pirk of Hoffmann-LaRoche, Inc.

§ The alpha estradiol benzoate was supplied through the courtesy of Dr. Kenneth W. Thompson of Roche-Organon, Inc.

16907 P

Prevention of Liver Necrosis following Ligation of Hepatic Artery.

J. Markowitz, A. Rappaport, and A. C. Scott. (Introduced by C. H. Best.)

From the Department of Physiology, University of Toronto.

In dogs, ligation of the hepatic artery is followed in approximately 100% of cases by death, as a result of a peculiar necrosis of the liver. In a series of experiments extending over many years we have tried to prevent this mishap by arterializing the blood flow of the portal vein. This year we appeared to be unusually successful, in that the animals survived such ligation. Examination at autopsy, however, always disclosed that the arterio-portal anastomosis was thrombosed, although the liver was grossly normal. During this year penicillin has been administered following the operations in one series of animals. Another series did not receive penicillin. The wholly unexpected finding was made, that when dogs with totally ligated hepatic arteries are given massive doses of penicillin, intraperitoneally and intramuscularly for one week, they usually survive, whether or not the gall bladder has been removed. The latter becomes gangrenous, often ruptures, but even this occurrence is well tolerated by the animal unless generalized bile peritonitis develops.

It is suggested that the immediate life-saving function of the hepatic artery is to maintain oxygen tension at a level incompatible with the proliferation of anaerobes constantly present in hepatic tissue.

16908

Relationship of Blood Sugar and Hypoproteinemia to Antibody Response in Diabetics.*

Michael G. Wohl, S. O. Waife, Stanley Green, and George B. Clough. (Introduced by E. Spiegel.)

From the Metabolic Division and Division of Biochemistry, Philadelphia General Hospital. Philadelphia, Pa.

Evidence has been presented that protein depleted animals as compared with normal controls possess little ability to manufacture antibodies. The poor antibody response in animals can be restored to normal by protein repletion.[1 3] We have reported on similar studies in man, particularly in the hypoproteinemic patient as seen in a large general hospital.[4]

The purpose of this paper is to direct attention to the fact that the diabetic with such complications as gangrene, osteomyelitis, etc., has a diminished antibody production; this low antibody response to antigenic stimulation appears to bear relationship to the blood protein values rather than to the fasting blood-sugar levels.

For this study a representative group of 64 diabetic patients attending the metabolic were generally irregularly rounded. The larger were often multinucleate. Unlike in the myometrium, the pigment inclusions in these connective tissue cells varied considerably in size. No pigment was present in the surface and glandular epithelium of the endometrium. These findings are in good agreement with previous histological studies of the uterus in the vitamin E-deficient rat.

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* Grateful acknowledgement is made of encouragement and supply of material by Dr. Gustav Martin and Mr. Steven Horochak of the Medical Research Department of the National Drug Company.

[1] Cannon, P. R., *J. Immunol.*, 1942, **44**, 107.

[2] Cannon, P. R., Chase, W. E., and Wissler, R. W., *J. Immunol.*, 1943, **47**, 133.

[3] Wissler, R. T., Woolridge, R. L., Steffee, C. H., Jr., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

[4] Wohl, M. G., Reinhold, J. G., Rose, S. B., Adams, L. A., Harvey, T., Francis, M., and Clough, G., *Arch. Int. Med.*, in press.

The uteri from 8 of 9 spayed animals maintained on the tocopherol-deficient diet exhibited little or no pigmentation of the myometrial cells and contained but few macrophages with pigment inclusions. The remaining animal in the group resembled the unspayed deficient animals in amount and distribution of pigmentation. Neither the unspayed nor spayed animals maintained on the tocopherol-supplemented diet showed microscopic evidence of abnormal uterine pigmentation.

Discussion. The present observations clearly indicate the uterine response to estrogen in vitamin E-deficient ovariectomized rats does not differ significantly from that of tocopherol-supplemented controls. This finding suggests that an intimate physiological relationship between the two substances is not very probable. It is possible, however, that conditions in the unovariectomized animal may be materially different in regard to the metabolism of estrogen and tocopherol.

Studies concerning the influence of vitamin E deficiency upon uterine pigmentation have been made by Mason and Emmel.[4] Unlike the present experiments, these authors did not find unequivocable evidence concerning the influence of ovariectomy upon the development of uterine pigment in their vitamin E-deficient rats. These contrary results may have been caused by the different experimental diets used.[‖] The fat content of the diet used by Mason and Emmel was double that of our diet and, in addition, their diet contained 2% cod liver oil. This is of particular significance since it has been shown that pigmentation in vitamin E-deficiency is intimately related to the intake of fat, especially if it contains large amounts of highly unsaturated compounds as does cod liver oil.[5] However, the apparent non-essentiality of the ovary in uterine pigment deposition in rats maintained on a high fat intake as compared with rats on low fat intake must remain unexplained at the present time.

The question may arise as to whether the absence of pigment in our vitamin E-deficient spayed animals might be due to the estrogen treatment just preceding sacrifice. This is not the case since, in other work not reported here, tocopherol-deficient spayed animals which had not received estrogen exhibited no macroscopic evidence of uterine pigmentation.

Summary. (1) Rats ovariectomized at weaning were maintained on a vitamin E-deficient diet for from 6 weeks to 10 months. No difference in the uterine weight response to injected estradiol benzoate was discernible between these animals and control animals of the same age maintained on the same diet supplemented by alpha-tocopherol acetate

(2) The uterine weight response to alpha-estradiol benzoate was significantly greater at 3 months of age than at 10 months regardless of tocopherol administration.

(3) Accumulation of acid-fast pigment in the uterus is characteristic of intact rats maintained on a vitamin E-deficient diet. Ovariectomy at weaning prevented the appearance of uterine pigmentation in animals maintained on the deficient diet for as long as 10 months.

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[4] Mason, Karl E., Dam, Henrik, and Granados, Humberto, *Anat. Rec.*, 1946, 94, 265.

[‖] It has been suggested by Dr. Karl E. Mason that the differences in the results may have been due partly to their use of Zenker's fixative whereas Bouin's fixative has been used in the present studies. For this and many other creative criticisms we are highly indebted to Dr. Mason.

[5] Mason, Karl E., and Emmel, Anna F., *Anat. Rec.*, 1945, 92, 1945.

TABLE I.
Relation of Protein Repletion (Positive N Balance) to Antibody Titre in Diabetics.

	Balance				
	Days	Avg g N/DA.	Max. titre	Avg alb.	Supplemented
			1:		
Ha.	20	7.5	40*	3.8	+
Ab.	36	6.6	640	4.7	+
We.	8	5.4	1280*	4.7	+
Cr.	36	4.9	320*	4.4	+
Pe.	74	4.7	5120	3.4	—
Sm.	62	4.4	320	3.1	+
Wat.	42	4.2	640	4.3	+
Cu.	55	2.6	2560	4.4	—
War.	49	—0.3	160	4.0	—

* Two injections typhoid.

TABLE II.
Average Serum Albumin Concentration.

	Day		
Group	0	17	27
Normal	4.87	4.85	4.80
Clinic diabetics			
I	5.27	5.48	5.37
II	4.28	4.18	4.40
Hospitalized diabetics			
(Supplemented)	3.11	3.77	3.87
(Non-supplemented)	3.31	3.41	3.41

3.11 and 3.31%. (See Table II). These figures remained essentially unchanged during the study except for the protein supplemented group whose albumin rose from a mean of 3.11 g to 3.87 g %.

The average highest titre of 16 diabetic patients with hypoproteinemia but nonsupplemented was 1:800 as compared with the average highest titre of 1:2900 in 9 normoproteinemic uncomplicated diabetics who only received 2 typhoid injections, whereas the hypoproteinemic group received 3 injections. There were 20 normoproteinemic clinic diabetics who also received 2 typhoid injections and whose highest titre was 1:1500 seven days after the second injection.

Nineteen hypoproteinemic patients were provided with oral supplements of protein in the form of lactalbumin hydrolysate or casein concentrate. The diets were carefully calculated and sufficient supplementary protein was added to double the daily protein intake as compared with that of the other hypoproteinemic group.

The results of this study showed an improvement in antibody response. During the first 10 days after initial injection the average titre in both supplemented and unsupplemented groups was approximately the same. Following each subsequent typhoid injection there was a greater rise in titre in the supplemented as compared with the nonsupplemented groups.

It is to be noted, however, that the best antibody response was obtained in the outpatient normoproteinemic diabetics (who were not suffering from an active complication of the disease) even though they received no protein supplementation.

The addition of extra protein to the diet did not appear to result in an increase in the severity of the diabetes during the experimental period of 30 days. There was no appreciable change in blood sugar levels, nor was there any change in plasma CO_2 combining power nor any increase in insulin requirements. It is of interest that there was no apparent relation between the degree of nitrogen storage and the peak of typhoid agglutinin titres (Table I), indicating that the retention of protein (as N) was not immediately reflected in an increase of antibody formation.

Whether the enhanced antibody-producing capacity is due specifically to an increase in the body's protein "pool" for globulin-antibody production or some other factor needs further study.

Summary. Sixty-four diabetic patients were studied immunologically and chemically. Nineteen diabetic patients with hypoproteinemia received nitrogen supplementation in the form of lactalbumin hydrolysate or casein concentrate. The following conclusions appear to be warranted: 1. The decreased capacity to produce antibody in the diabetic is apparently not related to hyperglycemia. 2. Diabetic patients with hypoproteinemia showed a lower average agglutination titre than those with normal blood protein values. 3. Oral supplementation with lactalbumin hydrolysate or casein concentrate enhanced antibody formation.

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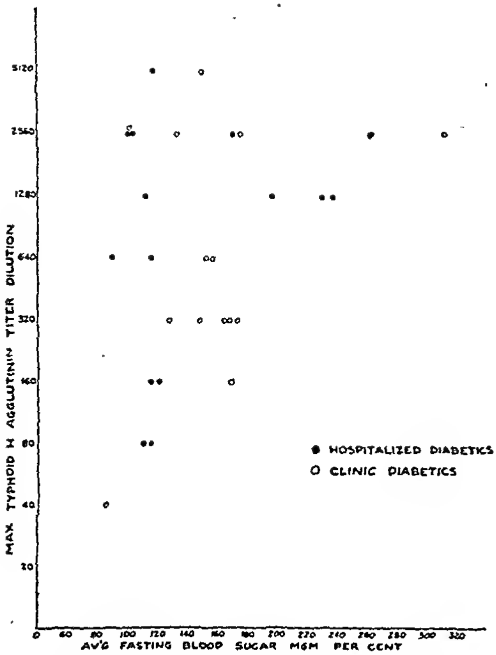

CHART 1.
Relation of the fasting venous blood sugar to antibody response.

clinic and housed in the metabolic wards of the Philadelphia General Hospital were selected. Eleven nondiabetic persons served as controls. Nineteen hypoproteinemic patients received protein supplementation in the form of lactalbumin hydrolysate or casein concentrate. The diet of the patients in the supplemented groups remained isocaloric with that of the nonsupplemented persons by reducing the fat intake by grams equivalent to the calories supplied by the additional protein. The average diet of diabetic men contained 2100 calories, of women 1800 calories.

The patients were studied chemically and immunologically by the methods previously described.[4] In addition, in 14 patients nitrogen balance studies were performed for a period of 36 to 55 days; detailed studies of the latter will be reported elsewhere. The data are plotted in Charts 1 and 2.

In Chart I the ordinates indicate the peak agglutination titre to typhoid H antigen; the abscissas indicate the corresponding average fasting blood sugar levels in mg %. Study of Chart I reveals that there is no relationship between the fasting blood sugar levels and the serum agglutinin content. To illustrate the patients with the highest titres (1:1280 to 1:5120) have fasting venous sugars from 100 to 300 mg %. On the other hand, titre levels of 1:160 or less were found in patients with average fasting venous blood sugars of 170 mg % or less. It would appear then that patients with hyperglycemia do not demonstrate a decreased capacity to produce antibody.

An analysis of Chart 2 shows that the diabetic patients with hypoproteinemia (serum albumin levels below 4 g %, the lower limit of normal for the method used) showed a lower average agglutination titre than the diabetic patients with normal blood protein values. The average serum albumin of the normal controls was 4.87 g %, of the clinic diabetic groups 5.27 and 4.28 g % respectively, of the 2 hospitalized diabetic groups

Typhoid H Agglutinin Titer in Diabetes Mellitus.

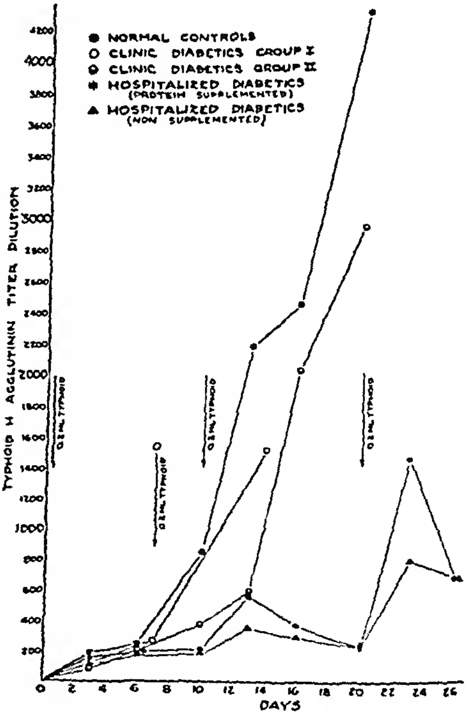

CHART 2.
Comparison of antibody formation in normal controls and diabetic adults with special reference to normoproteinemic and hypoproteinemic diabetics.

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16909

Streptocin, Antibiotic Isolated from Mycelium of *Streptomyces griseus*, Active Against *Trichomonas vaginalis*, and Certain Bacteria.*

Selman A. Waksman, Dale A. Harris, A. B. Kupferberg, H. O. Singher, and H. Styles.

From the New Jersey Agricultural Experiment Station, New Brunswick, N.J., and the Ortho Research Foundation, Raritan, N.J.

Schatz and Waksman[1] first reported that *Streptomyces griseus* produces, in addition to streptomycin, another antimicrobial substance. The latter was found to a small extent in the culture fluid but was present to a large extent in the mycelium, from which it was extracted with ether and chloroform. This crude substance was more active against the avian strain of *Mycobacterium tuberculosis* than against the human strain, the reverse being true for streptomycin. Further studies[2] showed that the material was soluble in various organic solvents, and was active largely against certain gram-positive bacteria, and only to a limited extent against the gram-negative organisms; it was later found to be active also against fungi, including both parasitic and saprophytic forms. One of the fractions of a similar extract isolated from a different strain of *S. griseus* was found to be trichomonadicidal. This substance can be designated as *streptocin.*

Whiffen, Bohonos and Emerson[3] reported the isolation from the culture medium of *S. griseus* of an antibiotic substance which is active against yeasts and fungi, and which is not active against bacteria. This antibiotic, designated as actidione, was soluble in organic solvents and in water, and was thermostable; it is distinctly different, however, from the trichomonadicidal substance which is reported here.

Isolation and antibacterial properties of crude preparation. Ether extracts obtained from the culture filtrate or from the mycelium of *S. griseus* gave the same antimicrobial spectra. The yields varied from 35 to 55 mg per liter of culture filtrate, and from 55 to 75 mg for the mycelium produced in one liter of medium. There was considerable variation, however, in the potency of the crude preparations depending on the strain of the organisms and on the conditions of growth. Much smaller amounts of the crude preparation were obtained from the submerged mycelium of two streptomycin-producing strains of *S. griseus* than from stationary pellicles, as shown in Table I. The stationary preparations were also more active than those obtained from the submerged mycelium (Table II).

By the turbidimetric method, it was established that 60 μg of the crude substance was sufficient to inhibit the growth of the pathogenic *M. tuberculosis* H37, including both the streptomycin-sensitive and resistant strains (Table III).

Isolation and purification of streptocin for trichomonadostatic and trichomonadicidal studies. For trichomonadostatic studies, a strain of *Trichomonas vaginalis* (No. 2) was used;[4] the organism was grown on a simplified trypticase-serum medium.[5] The method of inoculation and growth and the procedure employed in measuring growth are described

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* Journal Series paper, New Jersey Agricultural Experiment Station, Rutgers University; The State University of New Jersey, Department of Microbiology.

[1] Schatz, A., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 244.

[2] Waksman, S. A., Schatz, A., and Reilly, H. C., *J. Bact.*, 1946, **51**, 753.

[3] Whiffen, A. J., Bohonos, N., and Emerson, R. L., *J. Bact.*, 1946, **52**, 640; Leach, B. E., and Ford, J. H.. *J. Am. Chem. Soc.*, 1948, **70**, 1223; Whiffen, A. J., *J. Bact.*, 1948, **56**, 283.

[4] Johnson. G., Trussell, M., and Jahn, F., *Science*, 1945, **102**, 126.

[5] Kupferberg, A. B., Johnson, G., and Sprince, H., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 304.

TABLE I.
Production of Ether-soluble Antimicrobial Substances by 2 Strains of *S. griseus* Grown in Stationary and Submerged Cultures.

Strain of *S. griseus*	Culture	Yield,* mg	Activity, in dilution units, per 1 mg		
			S. aureus	*B. subtilis*	*B. mycoides*
3498	Stationary	131	65	114	114
3498	Submerged	31	9	16	16
3463-4	Stationary	318	27	47	47
3463-4	Submerged	38	13	13	13

* Per 5 liters of medium.

TABLE II.
Antimicrobial Activity of Ether-soluble Extract of *S. griseus*.
Dilution units per 1 mg of crude extract.

Organism	Activity
Staphylococcus aureus	120
Bacillus subtilis	120
B. mycoides	120
Mycobacterium phlei	15
Mycobacterium 607	4
M. tuberculosis H37Rv	2.4
M. tuberculosis H37RvR	2.4
M. avium	10.7
Escherichia coli	1
Proteus vulgaris	1
Serratia marcescens	1
Candida albicans	1
Trichophyton mentagrophytes	1.4

TABLE III.
Bacteriostatic Activity of Ether Extracts of *S. griseus* on *M. tuberculosis var. hominis.*

μg/ml of medium	Days of incubation			
	4	7	11	23
	Turbidimetric readings (in logs)			
	Streptomycin-sensitive strain H37Rv			
200	—*	—	—	—
100	—*	—	—	—
60	—*	—	—	—
20	0	0	2	14
10	0	3	11	90
6	0	5	14	83
2	0	4	14	90
0	1	5	17	80
	Streptomycin-resistant strain H37RvR			
200	—*	—	—	—
100	—*	—	—	—
60	—*	—	—	—
20	0	0	2	14
10	0	0	5	20
6	0	2	6	25
2	0	0	4	20
0	0	3	7	25

* A heavy precipitate formed but no growth occurred, as determined by plating procedures.

elsewhere.[6] To prevent bacterial contamination, 250 units per ml of medium of both streptomycin[7] and penicillin[4] were added to the suspension of the trichomonads, since these antibiotics had no effect either upon cell multiplication of the trichomonads or upon the activity of streptocin.

The streptocin used in the preliminary trichomonadostatic and trichomonadicidal studies was first isolated from a strain of *S. griseus* (No. 3533) which produced antibiotics active largely against gram-positive bacteria and trichomonads and was, therefore, neither streptomycin nor grisein. The culture was grown on a glucose-peptone-meat extract-NaCl-tap water medium in static or shaken condition. The mycelial growth was extracted in a Soxhlet with redistilled diethyl ether, some distilled water being added twice daily to the extraction chamber. The extraction was continued 24 hours after the ether became colorless. The ether-water mixture was removed, and redistilled acetone used for further extraction of the residual mycelium. This extraction was carried out as above. Only the fractionation of the ether-water extract is discussed in this paper.

The ether and water layers were separated, and the latter was reextracted with ether. The water then contained no trichomonadicidal activity and was discarded. The two ether fractions were combined, and the ether was removed under vacuum. The brown, oily

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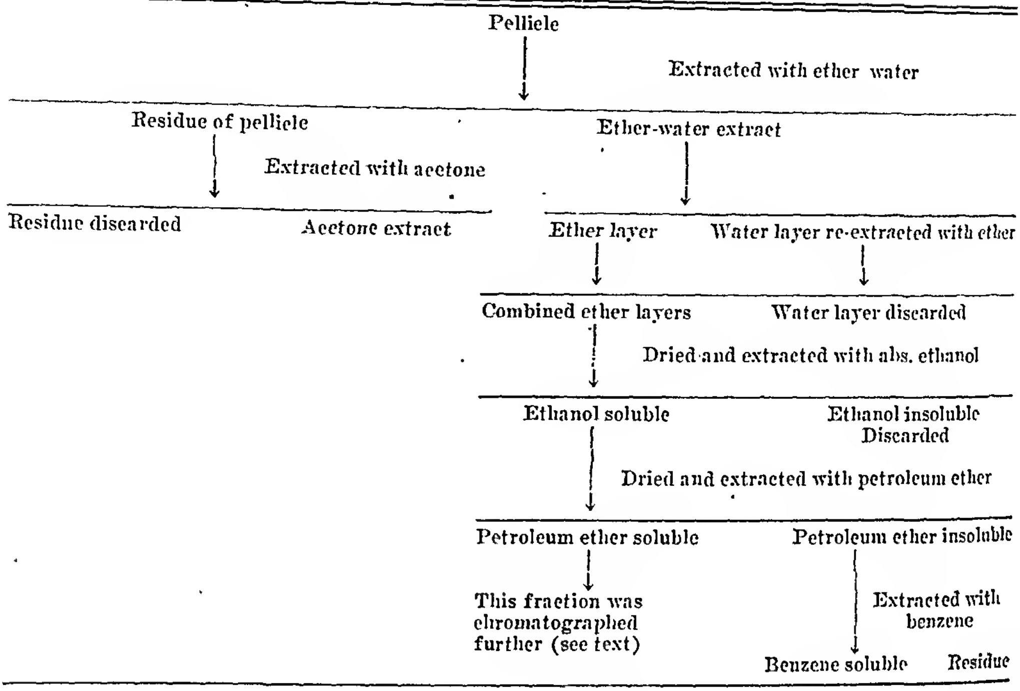
[6] Johnson, G., and Kupferberg, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, 67, 390.

[7] Trussell, R. E., *Trichomonas vaginalis and Trichomoniasis.* C. C. Thomas, Springfield, Ill., 1947, 61.

FIG. 1.
Extraction and Fractionation of Pellicle of *S. griseus* 3533.


Pellicle
Extracted with ether water
Residue of pellicle
Extracted with acetone
Residue discarded
Acetone extract
Ether-water extract
Ether layer
Water layer re-extracted with ether
Combined ether layers
Water layer discarded
Dried and extracted with abs. ethanol
Ethanol soluble
Ethanol insoluble Discarded
Dried and extracted with petroleum ether
Petroleum ether soluble
Petroleum ether insoluble
This fraction was chromatographed further (see text)
Extracted with benzene
Benzene soluble
Residue

residue was extracted with absolute ethanol. The ethanol-insoluble material was found to have no activity. The ethanol solution was distilled *in vacuo* and the resulting dry, yellow solid extracted with petroleum ether (b.p. 39-42°). The solvent was removed and the material dried over calcium chloride. The petroleum-ether-insoluble material was extracted with benzene; the benzene-soluble and -insoluble portions were dried *in vacuo*. The various steps used in this procedure are illustrated in Fig. 1.

The 3 fractions were chromatographed on columns of Brockman's Alumina (Merck). Since the general nature of the eluants of these columns was similar, only one of these, the petroleum-ether-soluble material, is discussed. This material was redissolved in dry petroleum ether and placed on the column in this solvent. The chromatogram was developed with increasing concentrations of benzene up to the pure solvent and then successively with acetone, ethanol, and methanol, until a 50-50 mixture of methanol-ethanol was reached. The material eluted at this point yielded, on evaporation of the solvent, an apparently crystalline material which possessed trichomonadicidal activity. Increasing concentrations of methanol were then employed for further development of the chromatogram. When 1% glacial acetic acid in methanol was used, more material was eluted. which on evaporation of the solvent, yielded a needle-like crystalline material which appeared in the form of rosettes. This preparation of streptocin had a high *in vitro* trichomonadicidal activity; its antibacterial spectrum is shown in Table IV. In contrast to the crude mixture isolated from the streptomycin-

TABLE IV.
Antimicrobial Activity of Crystalline Streptocin
Dilution Units per 1 mg of Streptocin.

Organism	Activity
E. coli	<3
B. mycoides	3
S. aureus	3
B. subtilis	75
M. ranae	<8
Mycobacterium 607	13
Mycobacterium 607R	13
M. avium	8

TABLE V.
Comparative Effects of Crystalline Streptocin and Actidione on Various Trichomonads.
Effects of 50 $\mu g/ml$ of antibiotics upon the trichomonad population after 48 hours incubation at 37°C, numbers* expressed as cells/mm^3.

Antibiotic	*T. foetus Br.*	*T. gallinae*	*T. vaginalis*
None	5,140	1,460	1,600
Streptocin	182	1,620	0
Actidione	1,490	0	10

* These figures represent the average of duplicate determinations.

TABLE VI.
Effect of Crystalline Streptocin Upon Cell Multiplication of *T. vaginalis.*

Conc. in units/ml in final medium	Population after 48 hr cells/mm^3
1.00*	0
0.66	10
0.14	1,010
0	1,600

* At this concentration, the agent is trichomonadicidal.

TABLE VII.
Effect of Sublimation Upon Yield of Streptocin from Crude Extract of *S. griseus* 3533.

	Trichomonadicidal units/mg
Crude ext.	0.96
Crude ext. sublimed	153
Residue from sublimation	0.77

producing strain of *S. griseus*[1,2] streptocin has a greater effect against the avirulent strain of *Mycobacterium* 607 than against the avian strain. It can be crystallized from 1% glacial acetic acid-methanol or from acetone but not from amyl acetate.

A trichomonadicidal unit is defined as that amount of material which when present in 1 ml of STS medium will kill the 75,000 seeded organisms in 48 hours' incubation at 37°C. A comparative study of streptocin and actidione upon different species of trichomonads is presented in Table V. The effect of different concentrations of *streptocin* upon *T. vaginalis* is shown in Table VI.

The active component can be sublimed at 100°C in a vacuum of 1 × 10^{-5} mm. This sublimation can be accomplished from the initial extract as shown in Table VII. Ac-

TABLE VIII.
Comparative Chemical and Biological Properties of Streptocin and Actidione.

Streptocin	Actidione (3)
Marked antibacterial activities	No antibacterial activity
Limited antifungal activities	Marked activity against fungi
Trichomonadicidal activity 1:29,000 against *T. vaginalis*	Trichomonadicidal activity <1:10,000 against *T. vaginalis*
No effect against *T. gallinae* at 50 $\mu g/ml$	Trichomonadicidal against *T. gallinae* at 50 $\mu g/ml$
Extremely low toxicity I.V. in mice	MLD_{50} I.V. in mice 150 mg/kg
Insoluble in $CHCl_3$	Soluble in $CHCl_3$
Forms gel with amyl acetate	Recrystallizes from amyl acetate
Stable at pH 11.0	Unstable to alkali
Sublimable	Nonsublimable

TABLE IX.
Antimicrobial Activity of Certain Chromotographic Fractions Other Than Streptocin.

	Dilution units per 1 mg			
Fraction	*E. coli*	*B. mycoides*	*S. aureus*	*B. subtilis*
10% acetone-benzene to 25% ethanol-acetone	1	24	75	100
Absolute ethanol	3	3	8	24
50% methanol-ethanol	3	3	3	100

tidione could not be sublimed under the same conditions. The crystalline material is soluble in water, methanol, and ethanol but not in chloroform. On standing, the material loses its crystalline structure. It is stable in a pH range of 2.0 to 11.0. It is stable to heat at 100°C for at least 60 minutes. The dry material is stable at 5°C for at least 4 months. Spectrophotometric measurements indicated no absorption maxima between 2200 and 6750 Å.

The comparative chemical and biological properties of streptocin and actidione are shown in Table VIII. These results show that streptocin is distinct from actidione. The antimicrobial spectrum of streptocin is similar to that of crude material isolated from the different strains of *S. griseus,* in that it acts largely upon gram-positive bacteria. However, other chromatographic fractions obtained from the crude material possess similar antimicrobial properties but are not trichomonadicidal. The antibiotic properties of several such fractions are shown in Table IX.

Course of production of streptocin by S. griseus 3533. The course of production of streptocin can be measured by its trichomonadicidal activities. Most of the material containing the trichomonadicidal activity is produced in the pellicles, and stationary cultures give much higher yields than submerged cultures. The results of typical experiments are shown in Fig. 2. Maximum production of the antibiotic is obtained in static cultures on the fifth day, when 76% of the activity is found in the pellicle and 24% in the culture filtrate.

The *Trichomonas foetus* used in these experiments was received from Dr. B. B. Morgan, University of Wisconsin. The authors of the New Brunswick group wish to acknowledge the assistance of Miss Dorothy Smith in testing the activity of the ether extract of pellicle of streptomycin-producing *S. griseus* against the various strains of *M. tuberculosis.* The authors of the Ortho group wish to acknowledge with appreciation the technical assistance of Mrs. Mary Williams.

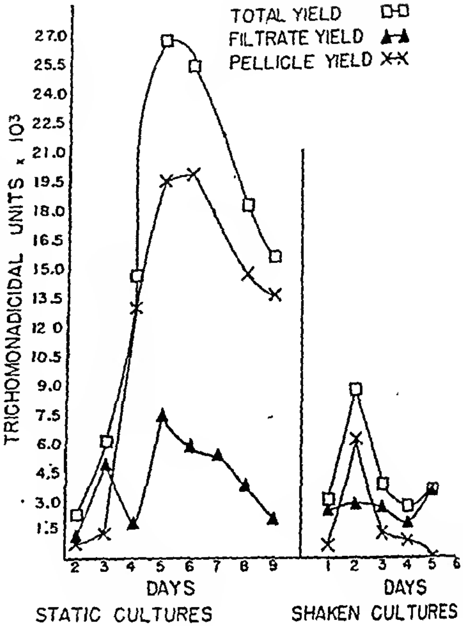


FIG. 2.

Comparison of relative yields of trichomonadicidal activity from the pellicle and filtrate of *S. griseus* No. 3533 grown in static and shaken cultures.

Summary. Several antibiotic substances were found in the ether-soluble extract of the mycelium of various cultures of *S. griseus,* which differed in their chemical nature and antimicrobial activities. One of these fractions from *S. griseus* No. 3533, designated as *streptocin,* possesses strong trichomonadicidal properties. It is active against gram-positive bacteria. Small amounts of streptocin are also present in the culture filtrate of the organism.

Streptocin is distinct from actidione in its physical and chemical properties and in the nature of its antimicrobial spectrum.

16910

An Experimental Study of Some Repository Dosage Forms of Penicillin.

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(Introduced by L. E. Arnow.)

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The injection of repository dosage forms of penicillin results in a prolongation of plasma penicillin concentrations. Crystalline sodium or calcium penicillin suspended in peanut oil and beeswax,[1,2] procaine penicillin suspended in oil[3,4] or in water[5] are used for this purpose.

Experimental. During the course of a research program designed to develop an effective suspending vehicle for such repository dosage forms, dogs were given an intramuscular injection of 300,000 units of penicillin in the form of some of these products or of an experimental preparation consisting of procaine penicillin in peanut oil and aluminum monostearate gel, as well as of the non-repository dosage form of sodium penicillin in aqueous solution. Penicillin concentrations of heparinized plasma samples were determined by a modification of the Rammelkamp tube dilution assay method.[6,7] For purposes of arithmetic averaging, amounts of penicillin not detectable by the assay were considered as zero values. Table I lists the average penicillin values found following the injection of each of these products into 5 dogs. It will be seen that, of the preparations tested, the use of the oil-monostearate suspension resulted in the greatest prolongation of detectable levels of plasma penicillin.

It was believed that a test of ability to prolong a period of protection against an experimentally induced infection might offer a more definitive means of evaluation of these preparations than the study of duration of detectable plasma penicillin concentrations. Accordingly, the mouse protection test described below was developed.

Mice were divided into groups corresponding to the number of preparations to be tested. At zero time each mouse received a single intramuscular injection in the left hind leg of 15,000 units (0.05 cc) of the appropriate penicillin suspension. At stated time intervals after this treatment, 10 or 20 mice from each of the original groups were challenged with 1,000 minimum lethal doses of a 6-hour culture of Pneumococcus Type I. Following challenge, the mice were observed for seven days, at the end of which time the final calculations of percentage survival were made.

The effect of penicillin particle size on protective ability was investigated because of the report that oil-beeswax preparations were most effective in prolonging plasma penicillin concentrations when large particle size crystals were used in the suspension.[8] Two preparations of sodium penicillin and an oil-beeswax base were formulated: in one the majority of the crystals measured 50 microns and in the other the majority of the particles measured less than 5 microns. Similarly, large and small particle size samples of procaine penicillin were suspended in oil and aluminum monostearate. Several tests were run on each preparation, and the reported figures represent

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[1] Romansky, M. J., and Rittman, G. F., *Science*, 1944, **100**, 196.

[2] Romansky, M. J., and Rittman, G. F., *N.E.J. Med.*, 1945, **233**, 577.

[3] Herrell, W. E., Nichols, D. R., and Heilman, Fordyce R., *Proc. Staff Meet. Mayo Clinic*, 1947, **22**, 567.

[4] Boger, W. P., Oritt, J. E., Israel, H. L., and Flippin, H. F., *Am. J. Med. Sciences*, 1948, **215**, 250.

[5] Whittlesey, P., and Hewitt, W. L., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 658.

[6] Rammelkamp, C. H., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 95.

[7] Miller, A. Kathrine, and Boger, W. P., *Am. J. Clin. Path.*, 1948, **18**, 421.

[8] Dowling, H. F., Romansky, M. J., Welch, H., Robinson, J. A., Chandler, V. L., Zeller, W. W., and Hirsch, H. L., *J.A.M.A.*, 1948, **135**, 567.

TABLE I.
Effect of Dosage Form on Average Plasma Penicillin Concentration in Dogs Following a Single Intramuscular Injection (1.0 cc) of 300,000 Units of Penicillin.

Dosage form		Avg plasma penicillin conc. (u/cc) at different hrs following penicillin injection				
Penicillin salt, 300,000 u	Suspending vehicle	1	8	24	72	96
Sodium	Water*	22	0.06	.01		
Procaine	Oil	3.8	1.7	.55	.02	
,,	Water	2.7	2.5	.19	.03	
,,	Oil-monostearate	2.8	1.0	.51	.12	.09

* Solution.

TABLE II.
Effect of Dosage Form on the % Survival of Mice Receiving a Single Intramuscular Injection (0.05 cc) of Penicillin and Challenged with Pneumococci.

Dosage form			Time (hrs after treatment) of challenge with 1,000 M.L.D. Type I pneumococcus — % survival									
Penicillin salt 15,000 u	Suspending vehicle	Particle size	1	4	8	24	48	72	96	120	144	168
Sodium	Water*		100	100	45	0						
Procaine	Oil		100	100	100	13	0					
,,	Water		100	100	75	7	0					
Sodium	Oil-beeswax	large				84	47	33	2			
		small				40	17	17	3			
Procaine	Oil-monostearate	large				66	30	3	0			
		small				100	97	87	66	34	13	0

* Solution.

calculations involving at last 20, and in most cases, 40 to 60 mice. Results of these experiments are included in Table II. It will be seen that none of the mice injected with aqueous solutions of penicillin were protected against an infection introduced 24 hours after the prophylactic treatment, but that 13% of the mice receiving procaine penicillin in oil and 7% of those receiving aqueous suspensions of the procaine salt retained sufficient penicillin for a 24-hour period to protect them against the pneumococcus infection given at that time. Large crystal sodium penicillin preparations protected a greater percentage of the test animals than did the small particle suspension, but, in contrast to these results, the small particle procaine penicillin in oil and aluminum monostearate was more effective than was the large crystal preparation, both in increasing the survival percentage and in prolonging the period of protection against infection. These data clearly demonstrate a remarkable superiority of small particle procaine penicillin suspended in oil and aluminum monostearate over all the other preparations tested in this manner.

Discussion. It is believed that the mouse protection test described here is a valid means of comparing the duration of therapeutic action of repository type penicillin preparations, and that it may be used to estimate the probable effectiveness of such preparations in producing prolonged plasma penicillin concentrations in human patients. Such a test has been found to be a useful tool in surveying experimental repository dosage formulas, and may be used as a guide in choosing preparations for clinical investigation.

Summary. A mouse protection test in which penicillin products are used prophylactically against subsequent challenge with Pneumococcus Type I is described. This test was used to evaluate repository type dosage forms of the antibiotic agent for their ability to prolong a period of protection against infection. Of the preparations tested the non-

repository aqueous solution of sodium penicillin was least effective, the suspensions of procaine penicillin in water or in oil were approximately equal in their activity, large particle size sodium penicillin in beeswax and oil was more effective, and small particle procaine penicillin suspended in oil and aluminum monostearate was the most effective preparation tested.

16911 P

Differentiation of Three Groups of Poliomyelitis Virus.*

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In a previous report in which 7 strains of poliomyelitis virus were compared by the method of challenging convalescent monkeys, Kessel and Pait[1] indicated that 4, BK, McK, Ca and Fr constituted one group, which was designated as Group A, or Group I.†

Two of the other 3 viruses studied, the La and Le, were distinct from the above 4 while the seventh, MV, appeared to exhibit an intermediate relationship. In 1946,[2] it was shown that La and Le were different by reciprocal neutralization tests.

Methods. In order to compare these relationships further, 4 of the viruses, McK, MV, La and Le were selected for reciprocal studies by 2 additional methods:

(a) challenge of vaccinated animals.

(b) Neutralization by pooled sera from the vaccinated animals. The vaccination was by the intramuscular route as recommended by Morgan,[3] the following schedule being used, 1.25 cc of 20% virus at 0, 1, 3 and 5 weeks, making a total of 1 g of infected monkey cord received by each animal. All animals were challenged with 100 P.D.$_{50}$ of homologous virus by the intracerebral route at the seventh week. All immune animals were then challenged with 100 P.D.$_{50}$ of a heterologous virus during the 13th week. Just prior to the heterologous challenge the monkeys were bled and their serums saved for neutralization

TABLE I.
Reciprocal Challenge of Vaccinated Animals. Numerator equals number of monkeys showing symptoms, denominator equals number of monkeys inoculated.

Vaccinated with	Challenged with			
	McK	MV	La	Le
McK	0/12	1/3	4/5	*
MV	4/4	0/12	1/4	5/5
La	5/5	*	0/11	6/6
Le	*	*	2/4	0/4

100 PD$_{50}$ used in challenge.
* Incomplete.

TABLE II.
Reciprocal Neutralization Tests.

Pooled serum from animals vaccinated with	Virus used in neutralization test			
	McK	MV	La	Le
McK	0/4	3/5	5/5	5/5
MV	4/4	0/4	0/4	4/4
La	5/5	2/4	0/5	4/5
Le	4/4	5/5	4/5	0/5
Controls	6/6	5/6	5/6	6/6

100 PD$_{50}$ of virus was used.

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* Aided by a grant from the National Foundation for Infantile Paralysis.

[1] Kessel, J. F., and Pait, Charles E., Proc. Soc. Exp. Biol. and Med., 1948, 68, 606.

† More recently 5 additional strains, Mi, from Dr. Thomas Francis, Jr.; Gu, isolated in Los Angeles; Ko, and Cp, from Dr. William M. D. Hammon; and Br, from Dr. David Bodian, have been tested and found to belong to Group I.

[2] Kessel, J. F., Moore, F. J., and Pait, Charles E., *Am. J. Hyg.*, 1946, 43, 82.

[3] Morgan, I. M., Howe, H. B., and Bodian, D., *Am. J. Hyg.*, 1947, 45, 379.

tests, a separate serum pool being made from monkeys vaccinated with each strain. In the neutralization test, the final virus concentration was 100 P.D.$_{50}$ per cc and serum dilution 1:2. This mixture was incubated for 4 hours in the refrigerator just prior to intracerebral inoculation of 5 monkeys with 1.0 ml each.

Results. Tables I and II summarize the results.

Conclusions. These results in conjunction with those of the previous study indicate that 3 groups of poliomyelitis virus have been demonstrated. I. One group encompasses at least 9 viruses compared in this study: Bk, McK, Ca., Fr, Mi, Gu, Ko, Cp. and Br. This group, which includes the greatest number tested to date has been designated tentatively as Group I. II. Another group so far comprises the La and MV viruses. III. A third virus, Le, differs sufficiently from the above two groups to represent a third group.

16912 P

Relationship of Body Specific Gravity to Body Fat and Water Content.*†

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It has been demonstrated by Behnke and his coworkers[1-3] that the specific gravity of the human body can be used as an index of the proportion of fat present. Rathbun and Pace[4,5] measured the specific gravity of eviscerated guinea pigs and also analyzed the bodies chemically for the fat content. They found that the body specific gravity of the animals decreased as the fat content increased, and that the range of specific gravity for the guinea pigs closely approximated that found by Behnke in his series of normal human males. The relationship between body specific gravity of the eviscerated guinea pig and the proportion of body fat was found to be a very exact one and agreed closely with the theoretical equation:

$$\% \text{ fat} = 100 \left(\frac{5.548}{\text{specific gravity}} - 5.044 \right)$$

The use of body specific gravity as an index of the amount of body fat in humans has been examined further in this laboratory. In an effort to describe more accurately the relationship which exists between metabolic rate and age, studies are being carried out to ascertain whether the "lean body mass," *i.e.*, body weight minus the fat content, may not be a better standard of reference for the metabolic functions of the body than surface area or weight of the body.

The difficulties encountered in weighing elderly people under water, a part of the procedure for measuring specific gravity of the body, were so great that it was decided to try to measure body water instead and calculate the lean body mass from this determination. A chemical method for the measurement of total body water, by the use of antipyrine (l-phenyl-2:3-dimethyl-5-pyrazolone) was then developed in this laboratory and the values obtained agreed closely with simultaneous measurements of body water by deuterium oxide.[6]

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

† Part of the expenses of this study were defrayed by the Josiah R. Macy, Jr. Foundation.

[1] Behnke, A. R., *Harvey Lecture Series*, 1941-1942, **37**, 198.

[2] Behnke, A. R., Feen, B. G., and Welham, W. C., *J.A.M.A.*, 1942, **118**, 495.

[3] Welham, W. C., and Behnke, A. R., *J.A.M.A.*, 1942, **118**, 498.

[4] Rathbun, E. N., and Pace, Nello, *J. Biol. Chem.*, 1945, **158**, 667.

[5] Morales, M. F., Rathbun, E. N., Smith, R. E., and Pace, Nello, *J. Biol. Chem.*, 1945, **158**, 677.

TABLE I.
Relationship of Body Specific Gravity, Body Fat, and Body Water.

Subject	Specific gravity	% body fat from spec. grav.	from antipyrine	% body water from spec. grav.	from antipyrine	% water from lean body mass
1	1.021	39.0	41.5	44.4	43.4	71
2	1.032	33.2	29.0	49.3	51.8	77
3	1.032	27.5	30.4	53.0	50.7	70
4	1.044	27.0	25.2	53.4	53.2	73
5	1.045	26.5	31.0	54.0	50.0	68
6	1.057	20.5	17.2	58.2	60.5	76
7	1.061	18.5	16.3	58.6	58.8	72
8	1.061	18.5	20.4	58.6	58.0	72
9	1.064	17.0	19.9	59.6	58.5	72

Thus in the same individuals it became possible to measure body water directly by a chemical method and by specific gravity, using Pace and Rathbun's formula.[7] This circumstance permitted a verification of both methods in a number of individuals. Pace *et al.* have previously shown a good check in one individual between the calculation of body water made from specific gravity and direct measurement by tritium oxide.[8]

Methods. The specific gravity of nine normal individuals was determined by weighing them in air and under water according to Behnke's procedure. On the same day, total body water of the subjects was measured by the use of antipyrine. In one individual, total body water was simultaneously determined by the use of both antipyrine and deuterium oxide, with good agreement by the two methods. Body fat and body water were then calculated or measured by two independent procedures.

Body fat was calculated from specific gravity[4,5] and from the antipyrine figure for total body water

$$\% \text{ fat} = 100\left(\text{body wt} - \frac{\text{wt body water}}{0.73}\right)$$

on the assumption that the defatted tissues of the body contain on the average, 73% water.

Body water values were ascertained by direct measurement with antipyrine and calculated from specific gravity

$$\% \text{ body water} = 100\left(4.424 - \frac{4.061}{\text{sp.gr.}}\right)$$ [7]

Results. The figures for per cent body fat calculated from specific gravity and from total body water determined by antipyrine agree fairly well (Table I) for the 9 individuals studied.

Likewise, there is good agreement of the values for per cent body water calculated from specific gravity and estimated chemically by the use of antipyrine (Table I).

In these tables the subjects are listed in order of increasing specific gravity. The inverse relationship of body water to body fat is readily noted; as the body specific gravity increases, total body water increases, while body fat decreases. Furthermore, the water content of the lean body mass is fairly constant.

Discussion. The fact that body specific gravity can be used to predict fat and water content in normal humans has received scant notice in the recent literature. The concept presented by Behnke and his co-workers that fat tissue is the chief variable of the body and that the fat-free tissue of the body or "lean body mass" is relatively constant in composition, is an important one. Variability in total body water depends, from this point of view, largely on the amount of body fat. This relationship seems not to have been widely appreciated.

In the literature, figures for the proportion of body water have run from 59.3% reported

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[6] Soberman, R., Brodie, B. B., Levy, B., Axelrod, J., Hollander, V., and Steele, J. M., *J. Biol. Chem.*, in press.

[7] Pace, Nello, and Rathbun, E. N., *J. Biol. Chem.*, 1945, **158**, 685.

[8] Pace, Nello, Kline, L., Schachman, H. D., and Harfenist, M., *J. Biol. Chem.*, 1947, **168**, 459.

as the result of a desiccation experiment[9] to 72.3 as measured by use of deuterium oxide.[10] The variations may, of course, have been due to the employment of different methods, but in the light of the present study, seem more likely to be the result of varying degrees of obesity.

These data serve to emphasize the necessity for taking into account the amount of adipose tissue of the body before comparison of the proportion of body water between individuals has much significance. They serve also to corroborate the usefulness of specific gravity as a measurement of the proportion of fat and water in the body.

Summary. 1. Body fat and water can be calculated from body specific gravity with considerable accuracy.

2. Use of an independent method of measuring body water clearly shows the close inverse relationship of per cent body fat and per cent body water.

3. The proportion of water in the body is highly variable unless it is expressed in terms of fat-free tissue (lean body mass.)

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[9] Gregersen, M. L., MacLeod's Physiology in Modern Medicine, 8th ed., 1938, p. 903.

[10] Moore, F. D., *Science*, 1946, **104**, 157.

16913

Assay of Aureomycin in Body Fluids: Observations on Individuals Receiving Aureomycin.*

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In order to place therapy with the promising new antibiotic, aureomycin, on a rational basis, the development of a satisfactory method of assay in body fluids is necessary. It is the purpose of the present communication to describe such a method of assay and to report some of the initial results from the application of this method in the study of the use of aureomycin.‡

At the time of the preparation of this report, only two groups of investigators have reported the results of attempts to assay the drug. The first group, using a *B. subtilis*-like organism, reported levels in humans following ingestion of various amounts of the drug.[1] The results were expressed as "units" which represented one-seventh of a microgram of the antibiotic. Paine, Collins, Finland, and Wells,[2-4] in studying aureomycin, reported on plasma levels of the drug as well as a quantitative method applicable to the urine. They stated that their method for determining the plasma levels was not satisfactory.[3,4] The urine levels were expressed as dilutions of urine which inhibited the test organism, streptococcus No. 98, rather than in terms of concentration of the drug.

Method. In this laboratory a modification of the Rammelkamp tube dilution technic[5]

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‡ The aureomycin used in this investigation was provided by Lederle Laboratories, Division of the American Cyanamid Company.

[1] Cox, H. R., personal communication.

[2] Paine, T. F., Collins, H. S., Finland, M., *J. Bact.*, 1948, **56**, 489.

[3] Collins, H. S., Wells, E. B., Paine, T. F., Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 174.

[4] Finland, M., Collins, H. S., and Paine, T. F., *J.A.M.A.*, 1948, **138**, 946.

[5] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

TABLE I.
Composition of Study Group Presented According to Route, Dose, and Period When Assay Was Done.

		Assays following initial dose		Assays following subsequent doses	
Route	Dose	No. persons	No. determinations	No. persons	No. determinations
Oral	0.16-0.6 g	1	1	5	10
	1.0 ”	11	36	13	35
	1.5-2.5 ”	2	8	2	7
I.V.	50 mg	9	47	2	4
	100 ”	1	3	1	5
I.M.	50 ”	5	12	—	—
	100 ”	1	4	2	4
	200 ”	1	1	—	—

which has been used for several years for the assay of penicillin and streptomycin in body fluids, has been applied to the assay of aureomycin. The method has proved practical and forms the basis for the present report.

Technic of the Test; Test Organism. The bacterium used in the test was a strain of beta hemolytic streptococcus (JB) which has been used in this laboratory for antibiotic assay for the past 5 years. This strain is sensitive to 0.0039 unit of penicillin per ml and to 0.015 μg of streptomycin per ml. A 24-hour culture of this organism in brain-heart infusion broth was centrifuged in a Hopkins tube[6] and then diluted with a buffer broth to be described below to make a final concentration of 4000 organisms per 0.5 ml of the broth.

Media. The broth used for the assay was a beef heart infusion (Difco) broth buffered with sodium phosphate to a pH of 7.6 and with 10% neopeptone. Ascitic fluid was added at the time of the test to a final concentration of 20%.

Serial Dilution of the Unknown Fluid. The serum or other body fluid was stored at about –40°C unless tested immediately. Fluids, such as urine, which were not sterile were filtered through Seitz pads. A series of 8 to 10 tubes were set up, each containing 0.2 ml of the buffer broth. To the first of these was added 0.2 ml of the material to be tested and serial 2-fold dilutions were made. The broth suspension of the test organism, in a volume of 0.5 ml, was then added to each tube, making a final volume of 0.7 ml. The tubes were well shaken and placed in an incubator at 37°C for 18 hours. The end-point of the test was taken as the highest dilution of the unknown material showing no growth visible to the unaided eye.

Control. With each assay, the test organism was set up with a known concentration of aureomycin which was prepared by diluting a standard solution of the drug. This standard solution contained a concentration of 10 μg by weight per ml and was stored between tests at –40°C. In the control set-up, 0.2 ml of this solution, containing 2 μg, was diluted in 2-fold steps with the buffer broth through 8 tubes. To each of these was added 0.5 ml of the test organism suspension. The final concentration of aureomycin in these tubes was therefore from 1.0 μg per ml down to 0.007 μg per ml. In this laboratory the standard organism used usually was inhibited in the tube containing 0.031 μg per ml and occasionally varied one tube in either direction, or from 0.062 μg per ml to 0.015 μg per ml.

As a further control over the technic, a known concentration of aureomycin, 5 μg per ml, was included in each run and was diluted as an unknown. In any run where this gravimetric value did not check with the bioassayed value, the results of the determinations on the unknown were considered inaccurate and the materials retested.

Example of Calculation. If, in determining the level of aureomycin in serum, the growth was inhibited up to a dilution of 1:8 and the control series showed the test organism to be inhibited at a concentration of 0.031

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[6] Todd, J. C., Sanford, A. H., Clinical Diagnosis by Laboratory Methods, W. B. Saunders, 1935, p. 703.

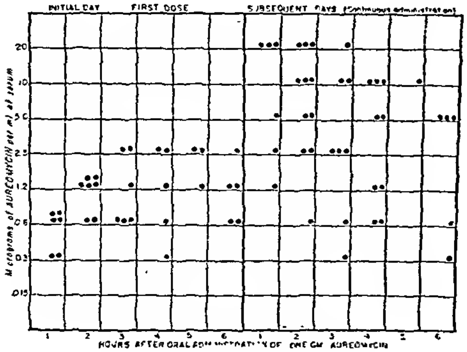

FIG. 1.

Graph illustrating blood aureomycin concentrations following an initial oral dose of one gram, as compared with blood levels following subsequent oral doses of one gram every 4 to 6 hours in patients receiving continuous therapy.

μg per ml, then the serum level was $8 \times 0.031 \times 5$ or 1.24 μg per ml of serum.

Study Group. Thirty individuals receiving aureomycin in a variety of doses and by several routes were studied in the present investigation, as presented in Table I. These individuals included patients under treatment for various infectious diseases and a group of normal individuals.

Results: Serum Levels Following Administration by Various Routes. Over the period of 6 months during which this method of assay has been used in this laboratory, certain general observations have been made. First, the test organism has not shown significant variation in its aureomycin sensitivity as determined by the control assay with known concentrations of the antibiotic. Retesting of serum specimens which had been stored over a period of 4 months at –40°C, showed no deterioration of the antibiotic content. No deterioration of the antibiotic in serum, plasma, or whole blood stored at 4°C for as long as 50 hours has been observed, although this possibility remains.[2] Therefore, all serum specimens were frozen at –40°C as soon as possible after being obtained.

Oral Route. Following the oral administration of an initial dose of 1.0 g of aureomycin in adults, significant concentrations of the drug were present in the blood within one hour, and the maximum concentrations were reached within 2 to 4 hours. The results of serial determinations on a group of 11 such individuals is presented in Fig. 1. The peak concentrations found in different individuals fell between 0.6 and 2.5 μg per ml of serum. Measurable amounts of the drug persisted in the serum for at least 6 hours, and in many individuals no decrease in concentration was observed at that time.

In most individuals to whom aureomycin was administered on a continuous schedule every 4 or 6 hours, the levels of the drug tended to increase gradually (Fig. 1). This was noted as early as after the second dose of the drug, but usually was more marked after several days. In some cases the later levels exceeded the earlier ones by many fold.

When the drug was given in doses of 0.5 or 1.5 g, the blood concentration did not appear to be affected to a degree proportional to the differences between these doses and the 1.0 g dose, although some difference was usually observed. Doses of aureomycin in children reduced roughly according to body weight appeared to produce concentrations comparable to those attained in adults following administration of 1.0 g.

Intravenous Route. Within 5 minutes following the intravenous administration of 50 mg of aureomycin dissolved in 5.0 ml of 0.784% Na_2CO_3 solution, drug concentrations were reached which were equal to or exceeded the maximum values obtained following oral administration of 1.0 g. Results of serial determinations of drug concentrations in 11 individuals following this dose are presented in Fig. 2. Serum levels rapidly declined dur-

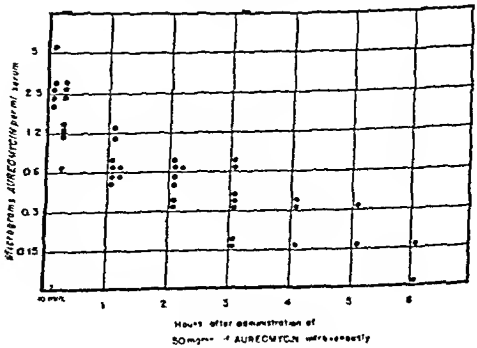

FIG. 2.

Graph illustrating blood aureomycin concentrations following the initial intravenous administration of 50 mg.

ing the first hour and decreased slowly thereafter. Measurable concentrations were present as long as 6 hours after administration.

Intramuscular Route. When the drug was administered by the intramuscular route in doses of 50 to 200 mg dissolved in 5.0 ml of a Sorensen phosphate buffer (pH 7.2) and mixed with an equal volume of 2% procaine, measurable levels were rarely noted during the following 4 hours. In only one of 21 determinations was a concentration greater than 0.15 µg per ml observed.

Urine Levels. Aureomycin concentrations were readily measured in the urine using this method of assay. Following doses of the order described above, concentrations of from 5 to 50 µg per ml were observed. Much smaller amounts were observed during the 4-hour period following the ingestion of 1.0 g than following the intravenous administration of 50 mg.

Cerebrospinal Fluid Levels. From 1 to 3 hours following the ingestion of 1 g, no measurable amount of aureomycin was observed in the cerebrospinal fluid in 3 assays on 2 individuals with meningeal irritation. In one normal child aureomycin was not demonstrated in the cerebrospinal fluid after the ingestion of 2 grams over a period of 24 hours.

Sensitivity of Organisms. The sensitivity of a variety of pathogens isolated in this laboratory varied from 0.017 µg per ml to 285 µg per ml. Most organisms tested were sensitive to less than 1.0 µg per ml.

Discussion. The tube dilution method of antibiotic assay in body fluids, although having certain disadvantages, represents a practical and convenient procedure. Variations in the end-point may occur with variation in the size of the inoculum of test organism.[2] These, however, are minimized by the use of a constant, small inoculum. Although the use of the inhibition of visible growth at 18 hours of incubation as the end-point may not coincide with complete inhibition as evidenced by subculture, neverthless, this discrepancy is relatively constant.

One of the problems inherent in any method of assay of an antibiotic is the rate of deterioration during handling and under conditions of incubation. This factor may be more important in the case of aureomycin than in the assay of penicillin and streptomycin.

Since high blood concentrations were often not reached for several days when the drug was given by mouth, a combination of the more efficient intravenous route and the more convenient oral route may be advisable in the treatment of many severe acute infections. The intramuscular route in our hands has been of very limited value because measurable serum levels were so rarely obtained. Frequent painful local reactions have occurred.

Summary. 1. A serial tube-dilution method of determinations of aureomycin concentrations in body fluids using an hemolytic streptococcus as the test organism is described.

2. Serum levels of aureomycin ranged from 0.3 µg to 2.5 µg during a 6-hour period following the initial oral dose of 1 g.

3. Serum levels or aureomycin following doses of 1 g on subsequent days in patients receiving continuous therapy ranged from 0.3 µg to 20 µg during a 6-hour period.

4. Serum concentrations of aureomycin following the intravenous administration of 50 mg ranged from 0.6 to 5 µg 5 minutes after injection and declined gradually over a 6-hour period.

16914

Hypothermia in the Mouse as a Bio-Assay of Endotoxin Protection Factor in Impure Penicillin.*

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From the Department of Medicine, University of Chicago.

In seeking an improved method of bioassay of the endotoxin protection factor of impure penicillin,[1-4] it was found, as reported by Beck[5] and Zahl and Hutner,[6] that mice given bacterial endotoxins. were unable to maintain their body temperature. In addition, we found that this hypothermia was less marked in animals treated with impure penicillin and that the degree of hypothermia was a function of the amount of impure penicillin administered.

Methods. Young adult (20 g) mice were kept in a constant temperature room at 24°C for at least 2 days before and during the entire time of each experiment. At the time the endotoxin was given the mice were placed in individual compartments which prevented them from huddling together and permitted the course of an individual mouse to be followed throughout the experiment.

0.5 ml of impure penicillin, containing 10,000 units of crystalline penicillin per ml, was given intraperitoneally at 20, 18 and 2 hours before the injection of the endotoxin.

The endotoxin, prepared from *Salmonella aertrycke* as previously described[7] was administered intraperitoneally as an endotoxin-saline mixture, the total volume of fluid being 1.0 ml in all cases.

TABLE I.
Method of Median Calculation.

Hr after endotoxin	22 hr	26 hr	28 hr	30 hr
	Protected mice			
	37.1	37.6	38.0	38.2
	36.9	37.5	37.7	37.7
	36.7	37.4	37.5	37.7
	36.3	37.0	37.4	37.3
	35.9	36.8	37.2	37.2
	35.6	36.6	36.8	36.7
	35.3*	35.8*	36.0*	36.6*
	34.4*	35.7*	35.8*	36.3*
	34.3*	35.2*	35.7*	36.0*
	33.3*	35.0*	35.6*	35.8*
	30.9*	32.3*	34.1*	34.7*
	30.7*	31.3*	32.8*	32.1*
Median temp.	35.4	36.2	36.4	36.7
	Unprotected mice			
	32.8	33.3	34.6	35.0
	32.3	32.7	33.2	33.4
	29.4	31.3	30.6	26.6
	27.9	27.4	27.5	26.5
	27.9	27.1	26.9	26.4
	27.4	26.5	26.5	26.1
	26.9*	26.4*	26.4*	25.9*
	26.5*	26.3*	26.3*	25.9*
	26.0*	25.3*	26.1*	25.5*
	25.8*	25.2*	25.2*	Dead*
	25.7*	Dead*	Dead*	Dead*
	25.6*	Dead*	Dead*	Dead*
Median temp.	27.1	26.5	26.5	26.0

* Mice with body temperature below the median of the group.

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† Research Fellow, U. S. Public Health Service.

‡ Douglas Smith Foundation Fellow in Medicine.

The impure penicillin was supplied through the courtesy of Dr. Robert D. Coghill of the Abbott Laboratories. The crystalline penicillin was supplied by Abbott Laboratories, Commercial Solvents Corporation, Lederle Laboratories, and Schenley Laboratories, Inc.

[1] Boor, A. K., and Miller, C. P., *Science*, 1945, **102**, 427.

[2] Miller, C. P., and Boor, A. K., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 18.

[3] Boor, A. K., Miller, C. P., and Hawk, W. D., *Fed. Proc.*, 1947, **6**, 240.

[4] Miller, C. P., Hawk, W. D., and Boor, A. K., *Science*, 1948, **107**, 118.

[5] Beck, L. V., A.A.A. S. Symposium on Approaches to Tumor Chemotherapy, 1947, p. 265.

[6] Zahl, P. A., and Hutner, S. H., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 156.

[7] Boor, A. K., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 168.

[8] Nielsen, E. T., *Acta Medica Scand. Supplementum*, 1938, **90**, 168.

The rectal temperatures were determined by the use of a copper-constantan thermocouple and a galvanometer. One junction was inserted a distance of 3 cm into the mouse's rectum and the precautions advised by Nielsen[8] regarding its position were observed. The other junction was placed in a constant temperature water bath.

As a routine schedule, 4 temperature determinations were made at approximately 21, 24, 27 and 30 hours after the endotoxin was injected. Less than 4 determinations increased the standard error. Twelve animals in each group were found to be sufficient for a fairly quantitative result, giving a standard error of about 0.8°C for the average of 4 determinations. This meant that a difference of approximately 3°C between the two groups of mice was significant.

The median temperature of each group of mice was chosen for the final calculation rather than the mean temperature because the loss of a mouse by death raised the mean temperature of the group. The dead mice were, therefore, listed at the bottom of their respective groups when the medians were determined and a more satisfactory computation resulted. This was felt to be a justifiable procedure since among 457 mice which died (of a total of about 1,500) 88% were animals with temperatures previously below and only 12% at or above the median. A sample experiment and calculation is given in Table I.

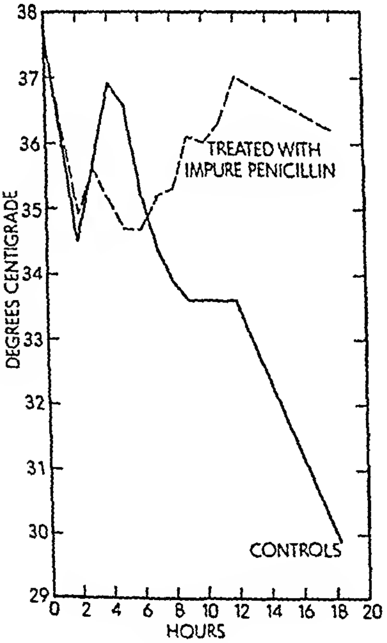

Fig. 1.

Median temperatures of mice injected with endotoxin.

Note the two phases of hypothermia after the injection of the endotoxin; the impure penicillin protects against the later more important phase of hypothermia.

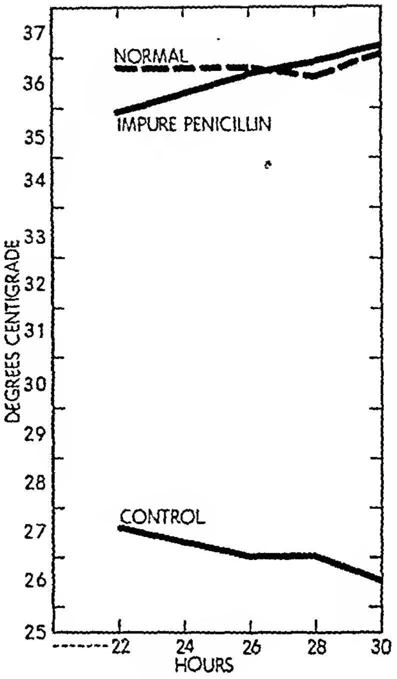

Fig. 2.

Median temperatures of mice injected with endotoxin.

Comparison of the median temperature of treated (impure penicillin) and control mice injected with endotoxin; and normals receiving no injection.

Experimental. Fig. 1 presents the results of a typical experiment. Two groups of mice were used: one protected by impure penicillin, the other untreated. Both groups were given 0.05 ml of the endotoxin intraperitoneally.

The results of an experiment using the routine procedure described under "methods" are shown in Fig. 2; the data appear in Table I. The temperatures of normal untreated mice kept under the same conditions and determined at the same time are also shown in this figure.

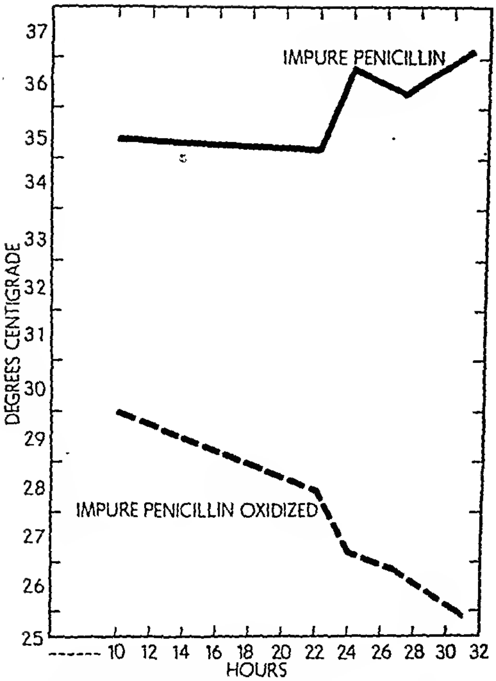

FIG. 3.

Median temperatures of mice injected with endotoxin.

Differences between protected and unprotected groups of mice as shown in Fig. 1 and 2 have been found in 25 separate experiments, using 1,140 mice in which temperatures were taken at least 4 times.

We have found a rough correlation between the degree of protection of a given substance computed by the LD_{50} and the anti-hypothermic effect. In order to investigate the correlation between the two types of experiments, the protective activity was destroyed by oxidation with hydrogen peroxide. The LD_{50} of the endotoxin in mice treated with oxidized impurity was 0.065 ml; in mice protected with unoxidized material the LD_{50} was 0.35 ml. The results of the two subsances in a temperature experiment are shown in Fig. 3.

The impure penicillin did not protect against the hypothermic effects of alpha naphthyl-thio-urea or crystalline insulin.

In order to obtain some quantitative results, an experiment consisting of 18 groups of 12 animals each was performed. The results can be seen in Fig. 4. This graph shows that the method can be used for a quantitative assay of the protective material; however, only a narrow range of the concentration of the protective material can be measured with one dose of endotoxin. In actual practice it was more economical of materials to use a constant dose of endotoxin and various dilutions of the protective factor. The curve shown in Fig. 5 plots the averages of 4 median temperatures of 4 groups of mice treated with varying concentrations of impure penicillin and injected with a standard dose of endotoxin.

Discussion. The greatest error in the method is the biological variation in the mice which necessitates the use of 12 animals in each group. The method also requires that the mice be kept in a constant temperature room and preferably in compartment cages to keep them separated from one another. It has the advantage of greater economy of mice and materials than a satisfactory determination of LD_{50} and is much more quantitative than the latter.

Autopsies, including microscopic examination, failed to disclose any significant trauma

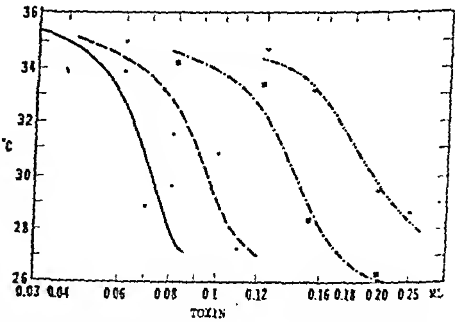

FIG. 4.

Median temperatures of mice injected with endotoxin.

This figure shows the effect of varying doses of *a standard preparation of* impure penicillin. Each point on the curves represents the average of 4 median temperature determinations between 20 and 30 hours after the administration of endotoxin.

—— Penicillin and saline treated controls.

– – – Treated with one part impure penicillin and 3 parts water.

–·–·– Impure penicillin diluted with an equal volume of water.

·–·–· Impure penicillin undiluted.

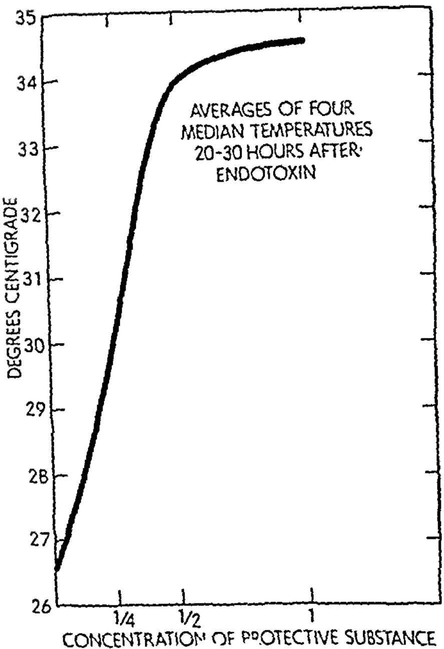

FIG. 5.

Effect of varying doses of impure penicillin. One can distinguish between the protective effect of penicillin and saline, impure penicillin diluted to ¼, and impure penicillin diluted to ½ by using 0.1 ml of endotoxin, as was done in this experiment. In order to distinguish half strength from undiluted material, one must dilute further.

from the insertion of the junction into the mouse.

Hypothermia may be regarded as the physiological response of the mouse to the endotoxin, comparable to fever in most other animals. That it is not merely an ante-mortem phenomenon is evidenced by the fact that less than one-third of the mice die in an experiment. In some instances mice have recovered after their temperatures have been as low as 26°C for 8 to 12 hours.

Summary and Conclusions. The endotoxin of *Salmonella aertrycke* injected intraperitoneally into mice caused a marked fall in body temperature. A certain phase of this hypothermia could be prevented to a significant degree by previous administration of impure penicillin. This phenomenon has been utilized for the quantitative assay of impure penicillin.

A correlation was demonstrated between the antilethal and the anti-hypothermic action of the impure penicillin against bacterial endotoxin in mice.

We are grateful to Dr. John F. Perkins of the Department of Physiology, University of Chicago, for the use of the thermocouple and galvanometer.

16915

Effect of Nicotinic Acid Monoethylamide on the Liver of the Young Rat.

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It has been reported by Brazda and Coulson[1] that a diet containing 1% coramine (nikethamide) produces a rapid and significant increase in the liver weight-body weight ratio of young rats. Nicotinamide, under similar conditions, produces no significant increase.[2] Inasmuch as coramine is disubstituted on the amide nitrogen whereas nicotinamide is unsubstituted it was decided to determine the effect produced by nicotinic acid monoethylamide in which the amide nitrogen is only monosubstituted. This communication reports the results obtained by use of this compound in the diet.

Experimental. The procedure employed was the same as that used in the study of the effect of coramine.[1] The nicotinic acid monoethylamide was mixed into the basic diet

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[1] Brazda, Fred G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1948, 67, 37.

[2] Coulson, R. A., and Brazda, Fred G., PROC. SOC. EXP. BIOL. AND MED., 1947, 65, 1.

TABLE I.*
Effect of the Ingestion of Nicotinic Acid Monoethylamide on the Liver of Young Rats.

No. days	Rat final wt	Liver wt wet	Liver wt dry	Liver wt as % body wt	Liver fat % wet wt	Liver fat % dry wt	Liver % solids	Gain in wt g/day
				Control				
5	54.7	2.72	0.80	4.98 ± 0.09[†]	3.87	13.27	29.21	+3.21
9	70.0	3.36	1.06	4.78 ± 0.09	4.22	13.38	31.47	+3.30
28	148.9	7.74	2.29	5.18 ± 0.13	3.22	10.89	29.60	+3.79
				1% nicotinic acid monoethylamide				
5	36.9	2.05	0.61	5.34 ± 0.12	4.28	14.28	29.53	+0.34
9	55.9	3.00	0.93	5.38 ± 0.10	5.14	16.58	30.91	+1.54
28	108.8	5.75	1.87	5.30 ± 0.11	4.50	13.65	32.78	+2.36

* Each figure in the table represents the average value obtained from 5 males and 5 females.

[†] $\text{P.E.} = \pm 0.6745\sqrt{\frac{\Sigma(v)^2}{n(n-1)}}$

at a level of 1%. Each of the 3 experimental groups consisted of 10 weanling rats, 5 males and 5 females, each from a different litter. One group was sacrificed for analysis at 5 days, a second at 9 days and the third at 28 days. The analytical procedures were identical with those reported previously.[1] The results appear in Table I.

Microscopic examination of hematoxylin-eosin preparations of sections of livers taken from the experimental animals yielded no unusual pathological findings. Binucleate cells and mitotic figures were seen in slightly greater numbers than in the control series. The difference was not great enough to be of true significance.

Discussion. The ingestion of nicotinic acid monoethylamide at a dietary level of 1% by the young rat failed to produce the significant increase in the liver weight-body weight ratio evoked by coramine.[1] It has been shown by Ellinger and Coulson[3] that coramine is converted quite slowly into nicotinamide in the human. Whether this involves de-ethylation or hydrolysis to nicotinic acid and subsequent conversion to nicotinamide is not known. It would appear from the results that the nicotinic acid monoethylamide is possibly more readily converted to nicotinamide than is coramine. That it is thus converted in the human has been demonstrated by Ellinger and Coulson.[3] However, since the main purpose of the experiment was to determine the effect of the compound on the liver no evaluations of the rate of conversion to nicotinamide were carried out.

Conclusion. Although the mechanism by which coramine produces its effect on liver size remains unknown it is quite apparent that disubstitution of the amide nitrogen is essential. Monosubstitution of the amide nitrogen with an ethyl group either cannot produce a significant effect or else this compound is so readily converted to nicotinamide, which has no such action, that an effective concentration of the necessary substance cannot be achieved.

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[3] Ellinger, P., and Coulson, R. A., *Biochem. J.*, 1944, **38**, 265.

16916

The Effect of Feeding Excess Glycine, L-Arginine, and DL-Methionine to Rats on a Casein Diet.*

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Previous reports[1-5] have demonstrated, that in rats and dogs, the addition of different levels of DL-methionine to a casein diet, affects the nitrogen balance index and may alter the physiological state of the animal. Low levels of methionine elevate the nitrogen balance index of casein, causing increased retention of nitrogen; intermediate levels do not alter the nitrogen balance index but cause weight loss and kidney hypertrophy; high levels depress the nitrogen balance index, there is a tearing down of body tissue, severe weight loss, kidney hypertrophy and an increase in creatine and creatinine excretion. It has also been shown[5] that excess L-arginine added to the high level DL-methionine diet, is effective in preventing, in large measure, kidney hypertrophy and also counteracts the lowering of the nitrogen balance index and weight loss.

The work described in this paper was designed to explore the effect of adding excess glycine alone and in combination with methionine and arginine, upon the nitrogen balance index of casein, organ and body weights, and creatine and creatinine excretion.

Methods. The methods for determining nitrogen balance indexes were the same as those described for the dog by Allison, Anderson, and Seeley[6] and for the rat by Brown.[4] These indexes (K) are functions of the rate at which the protein stores of the body are being filled by a given protein source and are calculated from the following equation: $NB = K\ (AN) - NE_0$ where NB is nitrogen balance, AN is absorbed nitrogen and NE_0 is the excretion of nitrogen on a protein-free diet. Mature, male rats of Sherman strain weighing approximately 250 g were used in the experiments. The rats were divided into 6 groups, 10 in a group and fed the following nitrogen sources. Values are on a dry weight basis.

Group	Casein
I	12% (controls)
II	12% + 4.8% DL-methionine
III	12% + 4.8% glycine
IV	12% + 4.8% glycine + 4.8% DL-methionine
V	12% + 4.8% glycine + 4.8% DL-methionine + 1.7% L-arginine
VI	12% + 4.8% glycine + 1.7% L-arginine.

The diets were, in addition, adequate with respect to carbohydrates, fats, minerals and vitamins. All the rats were pair fed with those fed excess methionine (Group II) since this group restricts their food intake. The experiment was continued for 20 days; the urine and feces were discarded for the first 4 days and then collected, thereafter, in four, 4-day periods. At the end of 20 days the animals were autopsied and the livers and kidneys dried at 95°C and analyzed for nitrogen. All deviations listed are standard errors.

Results and Discussion. In Table I are recorded the nitrogen intakes, the urinary and fecal nitrogen excretions, the nitrogen balances and the nitrogen balance indexes for the 6 groups.

The values for nitrogen balance index in

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* This work was supported by a grant from the Office of Naval Research and Development. Some of these data were included in a paper read before the Biological Division of the American Chemical Society, Washington, D.C., 1948.

[1] Miller, L. L., *J. Biol. Chem.*, 1944, **152**, 203.

[2] Allison, J. B., Anderson, J. A., and Seeley, R. D., *J. Nutrition*, 1947, **33**, 361.

[3] Brush, H., Willman, W., and Swanson, P., *J. Nutrition*, 1947, **33**, 389.

[4] Brown, J. H., Thesis, Rutgers University Library (1948), in preparation for publication.

[5] Brown, J. H., and Allison, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 196.

[6] Allison, J. B., Anderson, J. A., and Seeley, R. D., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 241.

TABLE I.
Nitrogen Intake and Excretion of Rats Fed Various Nitrogen Sources. Averages of 20 values obtained on 10 rats in each group.

Group	Ingested N mg/kg/day	Urinary N mg/kg/day	Fecal N mg/kg/day	N balance g N/kg/day	N balance index
I	397.4	250.2	108.1	+.039	.87
II	537.8	422.8	82.0	+.034	.59
III	600.8	434.0	114.6	+.052	.61
IV	721.5	596.1	96.0	+.029	.55
V	836.0	623.0	95.0	+.118	.49
VI	745.0	469.6	117.2	+.159	.64

TABLE II.
Weight Changes and Creatinine and Creatine Excretions of Rats Fed Various Nitrogen Sources. Average on 10 rats.

Group	Wt change (avg 20 days), g	Creatinine mg/kg/day	Creatine, mg/kg/day
I	7.4 ± 1.9*	25.8 ± 1.1	5.7 ± 1.0
II	—36.6 ± 6.1	28.9 ± 2.1	9.4 ± 1.6
III	+ 1.2 ± 3.6	26.7 ± 1.3	7.6 ± 0.5
IV	— 1.0 ± 3.6	30.5 ± 1.3	8.0 ± 0.9
V	— 5.0 ± 5.1	30.2 ± 1.2	12.5 ± 1.4
VI	— 5.1 ± 2.1	28.4 ± 1.1	10.3 ± 2.1

* Standard error.

Table I are calculated, relative to that of the casein fed controls (Group I). Examination of Table I shows that the addition of extra nitrogen in methionine (Group II), or glycine (Group III), or methionine plus glycine (Group IV) causes no significant change in the nitrogen balance. This may be interpreted as indicating that the nitrogen in these excess amino acids is not utilized under the conditions of this experiment. The drop in the nitrogen balance index for these groups approximately equals the index that may be calculated, assuming no utilization of the nitrogen in the excess amino acids fed. In Groups V and VI there is a significant rise in the nitrogen balance. The extra arginine fed to these 2 groups may be responsible for this rise. In these 2 groups, the nitrogen balance index calculated on the basis of no utilization of the amino acid nitrogen, is lower than that observed indicating, therefore, some usage of the excess amino acids. Previous studies indicate that excess arginine nitrogen added to casein is retained, in part, by the rat.[5] It should be pointed out that there appears to be a strain difference in the effects of excess methionine. For example, it has been found that 2.5% methionine, fed to Long-Evans strain rats, produced about the same effects in previous studies[4] as 4.8% produced in this one. Further work has shown that the addition of 7% methionine to the diet will produce negative nitrogen balance and tissue destruction in the Sherman strain rats.

The rats in all groups lost weight rapidly at first, and then more slowly during the first 6-8 days of the experiment (Table II). Thereafter, with the exception of those rats in Group II, a weight gain took place, so that at the end of 20 days the rats were nearly back to their original weights. The nitrogen balances paralleled the weight changes closely, being negative the first few days and even during the first collection period, but then positive for the last 12-14 days of the feeding. The rats receiving casein plus methionine (Group II), however, continued to lose weight throughout the 20 days, even though they were in positive nitrogen balance for the last 12 days.

In Fig. 1 is plotted the relationship between weight loss and nitrogen balance for the rats receiving casein alone (Group I). Fig. 2 illustrates the relationship between weight loss and nitrogen balance for Group II fed 12% casein plus 4.8% methionine. The loss in weight in the rats receiving excess methio-

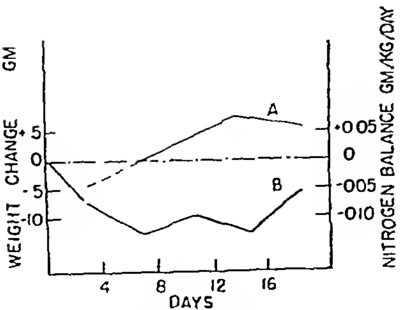

FIG. 1.
Relationship between weight loss and nitrogen balance for rats fed 12% casein.
Group I. Avg of 10 rats.
A. N balance B. Wt loss

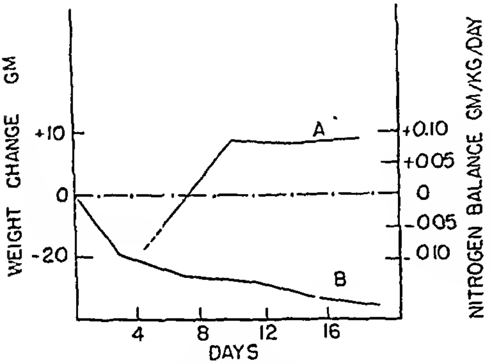

FIG. 2.
Relationship between weight loss and nitrogen balance for rats fed 12% casein + 4.8% *dl*-methionine.
Group II. Avg of 10 rats.
A. N balance B. Wt loss

nine while they were in positive nitrogen balance is associated with a loss in body fat. There is a marked lack of fat stores shown upon autopsy.[5]

The addition of glycine, or glycine plus arginine to the casein diet containing excess methionine counteracts the loss in weight caused by the excess methionine alone. Possibly glycine prevents the excessive lipotropic action of the methionine by aiding in its metabolism, or excretion. Based on previous studies,[5] larger amounts of methionine alone could cause continued loss in body nitrogen as well as fat, the animals never reaching positive nitrogen balance during the 20-day experimental period.

The excretions of creatine and creatinine are illustrated in Table II. The results are daily excretions and are averages of 20 determinations.

These data show that the addition of excess glycine to a casein diet does not significantly raise the creatinine or creatine excretion. The excretion of these materials is increased slightly by feeding excess methionine, alone, or in combination with glycine and arginine. Larger and more significant increases in the excretion of creatinine and creatine take place if more methionine is added to the diet since previous studies have demonstrated that excesses of methionine causing loss in body nitrogen will increase the excretion of creatinine.[5]

The effect of the diets on the organ weights is given in Table III.

The data in this table demonstrate that the addition of excess methionine to a casein diet causes an increase in liver and kidney weight and a slight increase in adrenal and thyroid weight; confirming previous results. The feeding of excess glycine causes an increase in liver weight but no other change. These data, together with those previously reported[5] demonstrate that both glycine and L-arginine antagonize in part the hypertrophic effect of the excess methionine.

That the increases in liver and kidney sizes were not due to increases in water content is demonstrated by the data in Table IV. The data in Table IV show that the addition of methionine, methionine plus glycine, or methionine plus arginine plus glycine, to a casein diet increases the nitrogen content of the kidneys. Glycine alone or in combination with arginine has no significant effect. Thus the hypertrophy of the kidney is associated with the presence of methionine.

Summary. In these experiments on rats, the addition of 4.8% glycine. 4.8% DL-methionine or 4.8% glycine plus 4.8% DL-methionine to a 12% casein diet did not alter the nitrogen balance as compared to controls receiving 12% casein alone but did reduce the nitrogen balance index, the excess amino nitrogen not contributing to the retention of nitrogen in the animal. The data indicates, on the other hand, that excess arginine (1.7%) contributes in part, to the retention of nitrogen. The addition of 4.8% glycine or

TABLE III.
Organ Weights of Rats Fed Various Nitrogen Sources.
Wet weights per 100 g of body weight. The results are averages obtained on 10 rats.

Group	Liver, g	Kidney, g	Adrenals, mg	Thyroid, mg	Testes, g	Seminal vesicles, g
I	2.66 ± .11	.617 ± .012	10.1	3.95 ± .26	.955	.310
II	3.15 ± .07	.856 ± .023	12.6	4.66 ± .19	1.07	.264
III	2.96 ± .05	.632 ± .014	8.8	4.01 ± .18	.912	.266
IV	2.91 ± .15	.707 ± .011	9.5	4.04 ± .07	.926	.312
V	2.79 ± .13	.788 ± .036	9.9	3.99 ± .10	.977	.228
VI	2.32 ± .11	.625 ± .016	9.1	3.88 ± .22	.960	.313

TABLE IV.
Water and Nitrogen Contents of Livers and Kidneys of Rats Fed Various Nitrogen Sources.
Averages on 10 rats.

	Water		Dry wt		Nitrogen		Total nitrogen	
Group	Liver %	Kidney %	Liver g/100 g B.W.	Kidney g/100 g B.W.	Liver %	Kidney %	Liver g/100 g B.W.	Kidney g/100 g B.W.
I	70.8	76.1	.815	.146	10.28	11.63	.084	.0169
II	70.9	76.4	.915	.201	10.23	11.61	.094	.0234
III	71.3	76.0	.846	.151	10.12	11.55	.086	.0174
IV	71.9	75.8	.794	.171	10.42	11.34	.083	.0194
V	70.3	76.9	.829	.181	10.53	11.16	.087	.0202
VI	70.7	76.0	.675	.149	10.86	11.29	.073	.0168

4.8% glycine plus 1.7% L-arginine to a casein diet containing excess methionine counteracted the weight loss and in part the kidney hypertrophy caused by the excess methionine. The slight increases in thyroid weights, associated with excess methionine, were also antagonized by glycine and arginine. Under the conditions of these experiments urinary creatinine and creatine excretion was not increased in rats by feeding excess glycine. The significance of these results is discussed.

16917

Therapeutic Effect of Choline Chloride in Dogs with Fat Emboli Produced by Bone Marrow Curettage.*

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Choline chloride as therapy for fat embolism was apparently used for the first time at Minneapolis General Hospital in 1948.

Moosnick, Schleicher,† and Peterson[1] observed that the fat content of human bone marrow is decreased 90% when a 1% choline chloride solution is given intravenously.

Methods. Twelve dogs were used to determine the value of choline chloride as a

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* Supported by a research grant from the Graduate School, University of Minnesota.

† The authors wish to express their thanks to Dr. Emil Schleicher for offering the suggestion that choline might be of value in treatment of fat embolism and for his invaluable advice. They also express thanks to Dr. Steven Barron of Department of Pathology for his advice and assistance in preparation of microscopic material.

[1] Moosnick, F. B., Schleicher, E. M., and Peterson, W. E., *J. Clin. Invest.*, 1945, **24**, 228.

therapeutic agent for fat embolism. Examinations of blood and urine were done to determine presence and size of fat globules.

Fat embolism was produced in 12 dogs according to Friesen *et al.*,[2] under pentobarbital anesthesia by doubly drilling through both cortices of the lower ⅓ of the humerus, and lacerating the bone marrow with a sharp curet. These dogs were observed for a period of 5 days, and then autopsied.

After surgery half the dogs were given .25 cc 1% choline chloride per kilogram body weight intravenously 3 times a day at 5 hour intervals into the leg vein opposite the traumatized humerus. The other half served as controls.

Blood specimens were collected daily from each dog.

Daily specimens of urine were collected by catheter. Five cc of urine was centrifuged for 5 minutes. One drop of urine from the meniscus was placed on a glass slide and one drop Sudan III was added. The preparation was then examined under high power after standing for five minutes. The size and number of fat globules were recorded.

Results. In the choline treated dogs, fat globules were found in the blood plasma between the second and fourth day and these varied from 4 to 12 micra. Three globules were the maximum per field. None was observed in blood plasma on the fifth day.

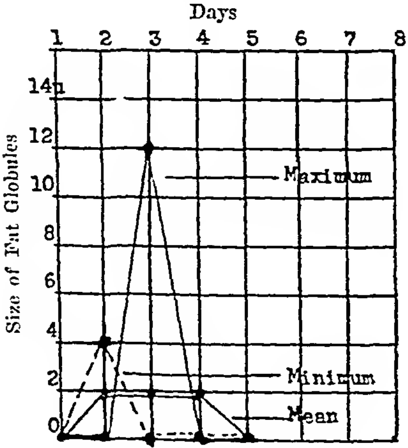

FIG. 1.
Fat in Blood of Choline Dogs.

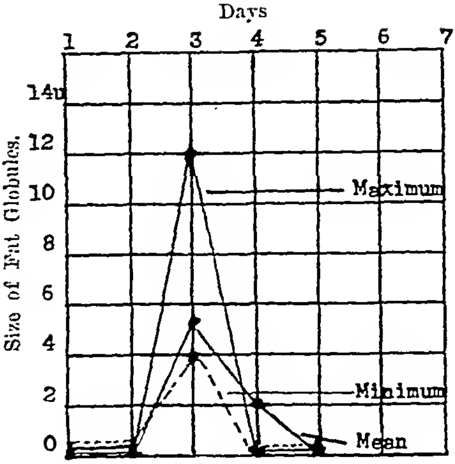

FIG. 2.
Fat in Urine of Choline Dogs.

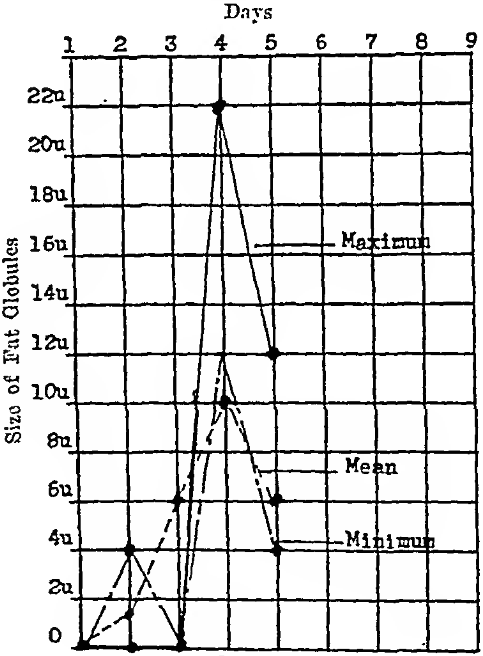

FIG. 3.
Fat in Blood of Control Dogs.

Fat globules appeared in the urine of the dogs treated with choline on the third and fourth day after surgery, varying from 4 to 12 micra in diameter. but not more than 2 fat globules per field were noted. None was seen in the urine on the fifth day.

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[2] Friesen, S. R., Merendino, A. K., Baronofsky, Ivan, Mears, Frederick B., and Wangensteen, O. H., *Surg.*, 1948, 24, 148.

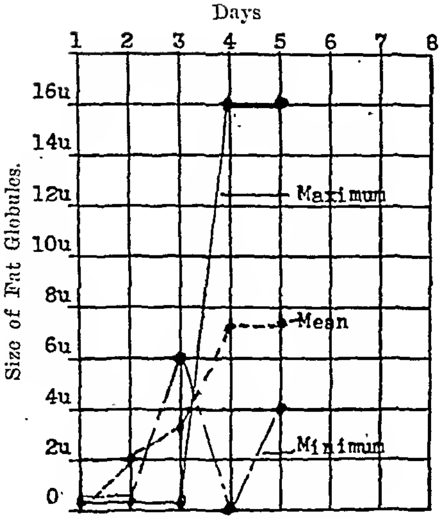

FIG. 4.
Fat in Urine of Control Dogs.

In the control dogs, fat globules were present in the urine and blood plasma from 2nd day after surgery to the time of autopsy. Maximum fat was present from the third day on. The size of fat globules varied from 4 to 20 micra; many were seen per high power field.

After autopsy, formalin fixed, hematoxylin and eosin stained sections of lungs showed fat emboli in all dogs with no appreciable difference between treated animals and controls.

Conclusion. Choline appears on the basis of plasma and urine examinations to have been of value in the management of experimentally produced fat embolism in dogs. Lung sections at five days after trauma proved inconclusive.

Note. Choline has been used in clinical cases of fat embolism with apparently very striking results. This is to be reported.

16918 P

Acute Occlusion by Ligature of the Portal Vein in the *Macacus rhesus* Monkey.*

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From the Department of Surgery of the New York Hospital, and Cornell University Medical College.

It is commonly accepted today that sudden occlusion of the portal vein both in the human and in animals is followed within a matter of minutes to hours by death. The first demonstration of this phenomenon is credited to Oré,[1] who noted that rabbits in which the portal vein was ligated survived but a short time. Since this original observation a large number of confirmatory reports can readily be found. The most definitive study is that of Elman and Cole,[3] who proved not only that ligature of the portal vein in dogs and cats is followed promptly by the death of the animal but also that failure to survive the procedure was due to loss of effective circulating blood volume secondary to pooling within the obstructed splanchnic venous bed. In addition to animal experiments such as these, there are a number of reports in the literature of occlusion of the portal vein in the human by thrombosis, either septic or bland, and by ligature made necessary either in the course of a surgical operation or secondary to intra-abdominal trauma.

The conclusion drawn from the animal experiments is that sudden obstruction of the portal vein is incompatible with life; from a review of the phenomenon as it is reported in the human the implication is also that in general a patient cannot survive sudden portal occlusion. A few reports are available for study, however, which indicate that occasionally the so-called "hepatopetal" veins may be sufficiently well developed to take over im-

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* Supported by U. S. Public Health Service grant.

[1] Quoted by Neuhof.[2]

[2] Neuhof, Harold, *S. G. and O.*, 1913, **16**, 481.

[3] Elman, R., and Cole, W. H., *Arch. Surg.*, 1934, **28**, 1166.

mediately the burden of returning sufficient portal blood to the systemic circulation to prevent shock and death.[4] As far as can be determined, however, it has never been conclusively settled whether or not the human can survive sudden occlusion of the portal vein by ligature. Because occasionally a patient with pancreaticoduodenal cancer must be refused radical resection because of invasion of the portal or superior mesenteric veins, it has recently become important to decide whether or not resection of these structures is compatible with life and prolonged survival. Today it is generally accepted that whatever else is done during radical pancreaticoduodenectomy the portal and superior mesenteric veins must preserved.

In an effort to elucidate this problem further, its study has been undertaken in the *Macacus rhesus* monkey. This animal was selected because the anatomy of the portal, superior mesenteric, and splenic veins is nearly identical with that of the human. Furthermore, the anatomical relationships of the pancreas and duodenum approximate the retroperitoneal position of these structures found in man and not present in dogs, cats, rabbits, etc.

Experimental Procedure. Adult *Macacus rhesus* monkeys were operated upon under open drop ether anesthesia and various segments of the portal circulation occluded by double ligature with medium silk. The animals recovered promptly from anesthesia and, except for the discomfort apparently due to the celiotomy wound, gave no evidence of having been disturbed by the procedure. They are all surviving, the postoperative period varying from 16 days to 6 weeks. In an effort to document further these experiments, all of the animals have been subjected to portal venography with 2-4 cc of thorium dioxide injected into mesenteric venous channels at about the level of the mid-jejunum. All of the venograms (for representative venograms see Plates I and II) have demonstrated complete occlusion of the portal vein, blood gaining access to the systemic circulation by various routes, chiefly, however, the right and left colic veins and the small retroperitoneal vessels in the region of the head of the pan-

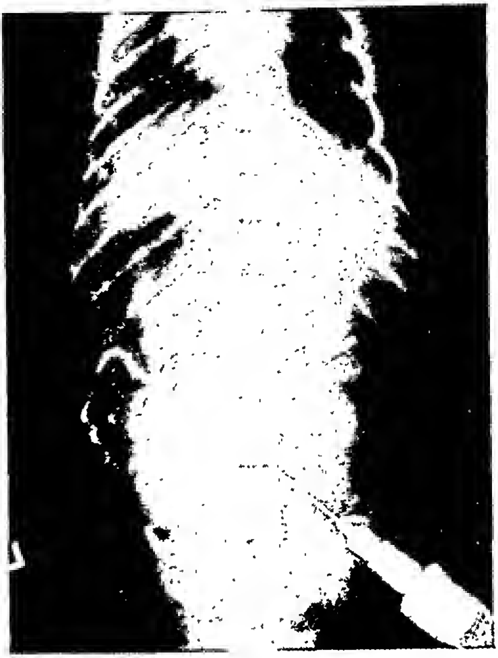

PLATE 1.
Experiment No. 4. Splanchnic venogram (3 cc thorium dioxide) 13 days following ligation of the superior mesenteric vein.

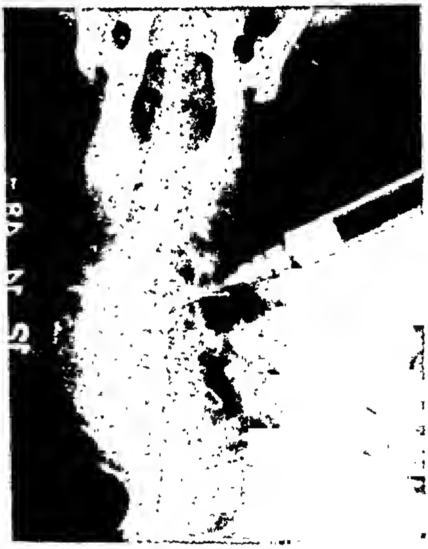

PLATE 2.
Experiment No. 6. Splanchnic venogram (3 cc thorium dioxide) 6 days following ligation of portal, superior mesenteric, and splenic veins.

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[4] Colp, Ralph, *S. G. and O.*, 1926, 43, 627.

TABLE I.
Acute Occlusion by Ligature of the Portal Vein in the *Macacus rhesus* Monkey.*
All recovered and present condition is normal.

Exper. No.	Vessels ligated	Sex	Days post-op.	Major collateral circulation
1	Portal vein	F	51	Splenic vein Inferior mesenteric vein Middle colic vein
2	” ”	M	50	Same
3	” ”	M	49	Same
4	Superior mesenteric vein	F	43	Middle colic vein. Retroperitoneal vein Hepatic ligament vein
5	Portal vein Superior mesenteric vein	F	38	Middle colic vein Inferior mesenteric vein
6	Portal vein Superior mesenteric vein Splenic vein	F	23	Retroperitoneal vein Middle colic vein Omental vein
7	Same	F	16	Same

* Seven *Macacus rhesus* monkeys subjected to ligation of various segments of the portal venous system. Survival periods and chief systemic pathways of anastomosis are indicated in the column headed "*Major Collateral Circulation.*"

creas. To date none of the animals has developed clinically detectable nutritional deficiencies or ascites. Table I outlines the seven experiments performed.

Summary and conclusions. Various segments of the portal circulation (including the portal vein) have been suddenly occluded by ligature in 7 adult *Macacus rhesus* monkeys. All of the animals have survived this procedure uneventfully and are in apparent good health from 16 to 51 days postoperatively. By means of portal venography anastomotic channels have been demonstrated by way of which blood is immediately returned to the systemic circulation in sufficient quantities to prevent these animals from succumbing to shock due to depletion of their circulating blood volume.

16919

The Ulcer-Inhibiting Action of Pyrogens.

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A series of urinary extracts under investigation for their ulcer-inhibiting action in the Shay rat, were found to contain a high concentration of pyrogens. Since Necheles[1] had shown that pyrogens suppressed gastric motility in dogs, it seemed desirable to study the inhibitory effect of purified pyrogens in the pylorus-ligated rat. Pyrogens prepared from cultures of *B. prodigiosus, Pseudomonas aeruginosa* and *E. typhi* were investigated.

Method. The assay procedure for estimating anti-ulcer activity was essentially that of Pauls, Wick, and MacKay.[2] Sprague-Dawley male rats of 150-170 g weight were fasted for 48 hours in cages with coarse mesh bottoms. Water was given *ad libitum.* After ether anesthesia, the duodenum was exposed, grasped lightly with fine forceps and ligated at the pyloric sphincter with braided silk thread. The incision was closed with metal clips and painted with collodion solution in order to avoid ingestion of blood residue from the wound.

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[1] Necheles, H., *Am. J. Physiol.*, 1942, **137**, 28.

[2] Pauls, F., Wick, A. N., and MacKay, E. M., *Gastroenterology*, 1947, **8**, 774.

TABLE I.

Pyrogen	M.P.D.,* µg per kg	Nitrogen, %	Reducing substances, %	Glycosamine, %
B. prodigiosus	.005	.7	68.9	2.5
Pseudomonas aerugenosa	.02	1.1	64.5	4.2
E. typhi	.01	.8	58.7	3.1

* M.P.D. or minimal pyrogen dose refers to the minimal amount which will cause a temperature rise of 0.6°C in rabbits (U.S.P. XIII).

TABLE II.

Preparation	Dose range, µg	Route	Total No. rats	No. ulcerated	Ulcer index
Controls	—	Intrav.	56	53	315
Prodigiosus pyrogen	3-10	”	28	10	72
” ”	15-20	”	24	7	55
” ”	25-30	”	30	5	31
” ”	35-50	”	20	1	15
” ”	10-25	Intraper.	15	8	140
” ”	50	”	10	2	80
” ”	100-1000	”	15	0	0
” ”	100-1000	Oral	3	3	367
Pseudomonas pyrogen	10-50	Intrav.	29	8	52
” ”	100-500	”	11	0	0
Typhoid pyrogen	50-250	”	8	2	25

Pyrogens were dissolved in pyrogen-free dilute buffer and injected in 0.5 ml volumes intravenously in a tail vein or intraperitoneally just prior to pyloric ligation. In one experiment, pyrogen solution was given orally by stomach tube a few minutes following the operation when recovery from anesthesia was nearly complete. Control rats received 0.5 ml pyrogen-free buffer solution intravenously. Animals were sacrificed at the end of eight hours. Volume and free acidity of stomach contents were measured and recorded and the degree of ulceration scored using an arbitrary scale of +4 to +1 according to the number and size of lesions observed. Zero was recorded when no macroscopic abnormality was seen. The average score for each group was multiplied by 100 to give an "ulcer index" following the method of Risley, Raymond, and Barnes.[3]

Pyrogens. Pyrogens were prepared from pure cultures grown on synthetic medium[4] and isolated by a procedure to be described in a subsequent publication. Full analytical data on these pyrogens will appear later.[5] However, Table I gives the pertinent information on each of these highly purified preparations used in the experiments reported here.

Results. Experimental results are recorded in Table II which for brevity is tabulated in dose ranges of administered pyrogens rather than for separate dosages.

Among 56 control rats not treated with pyrogen, 53 showed ulceration at the end of 8 hours to an average degree of +3.15 or an ulcer index of 315. Intravenous doses of 3-10 µg prodigiosus pyrogen protected 18 of 28 rats and reduced the ulcer index to 72. Increasing doses increased the number of rats protected and reduced the ulcer index to a low of 15 at 35-50 µg doses. Intraperitoneally, corresponding amounts of this pyrogen are somewhat less effective than when given intravenously. However, doses ranging from 100-1000 µg offer complete protection, none of the 15 rats treated with this amount of pyrogen showing any ulceration. Orally this pyrogen appears to be completely lacking in protective activity against ulceration. The data, however, are too limited to allow generalization with respect to this mode of administration.

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[3] Risley, E. A., Raymond, W. B., and Barnes, R. H., *Am. J. Physiol.*, 1947, **150**, 754.

[4] Rodney, G., and Welcke, M., *J. Bact.*, 1945, **50**, 129.

[5] Rodney, G., and Devlin, H. B., to be published.

Pseudomonas pyrogen was used in only 2 dose ranges of 10-50 μg in 29 rats and 100-500 μg in 11 rats. Critical examination of the individual protocols indicates that this pyrogen had slightly less inhibitory effect on ulceration than the prodigiosus pyrogen which correlated with the comparatively low pyrogenic activity as measured in the rabbit test.

Typhoid pyrogen was used only in the larger dose range, two of eight rats showing minor ulceration with this preparation.

Not shown in the table but deserving of mention are results with a non-pyrogenic polysaccharide prepared by the pyrogen isolation technic from pneumococcus type II cultures. This substance showed no anti-ulcer activity in 4 rats in doses up to 200 μg.

Also omitted are detailed data on gastric volume and free acidity. However, it may be stated that among all rats studied in these and related experiments, the mean gastric volume of 208 ulcerated rats was 8.9 ± 2.6 (S.D.) ml whereas in 94 non-ulcerated rats secretion amounted to 5.9 ± 3.2 ml. Likewise free acidity of gastric contents from ulcerated rats was equivalent to 5.3 ± 2.4 ml N/10 NaOH. Non-ulcerated rats showed values of 3.0 ± 2.3 ml. Among both groups of data the differences between the means from ulcerated and non-ulcerated rats are statistically significant.

Conclusion. As judged by response in the Shay rat, pyrogens from *B. prodigiosus, Pseudomonas aerugenosa* and *E. typhi* are highly active ulcer-inhibiting substances. Whether the presence of these substances in various tissue and urinary extracts may account for the reported anti-ulcer activity of these preparations remains to be investigated. The mode of action of pyrogens is not clear but may be associated with the obviously diminished acidity and volume of gastric secretion which occurs as a result of administration of these substances. There was observed no evidence, however, of any general "toxicity" in the animals which received purified pyrogens.

Summary. Intravenously administered purified pyrogens from cultures of *B. prodigiosus, Pseudomonas aeruginosa* and *E. typhi* possessed marked ulcer-inhibiting action in the Shay rat.

16920

Increased Requirement for Pteroyl Glutamic Acid During Lactation.*

MARTIN B. WILLIAMSON. (Introduced by J. J. Smith.)

From the Department of Biological Chemistry, Loyola Medical School, Chicago, and the Harvard Medical School, Boston.

A series of experiments had been undertaken in an attempt to produce an anemia in fetal and newborn rats. One of the technics employed was the feeding of succinyl sulfathiazole (SS) to the maternal rats. During the course of the study, it was observed that the newborn rats from mothers receiving the diet containing SS, died within five days after birth. It was thought that this high mortality among the newborn might be due to either a toxic effect or to a failure of lactation.

Preliminary experiments indicated that SS or its hydrolysis products did not have any toxic effect on the newborn rats. Although SS passes through the alimentary tract substantially unchanged and is not absorbed, some small part of it is hydrolyzed to sulfathiazole which may be absorbed. On a diet containing 1% SS, rats were found to have a blood level of approximately 0.3 mg % sulfathiazole.[1] The administration of SS or

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* This work was supported in part by a grant from the Foundation for Vision for the study of Retrolental Fibroplasia.

TABLE I.
Effect of Diet of Pregnant Rats on the Survival Time of the Newborn.

Diet	No. of litters	No. of newborn	Birth wt, g	Average survival, days	No. of newborn surviving 20 days	% mortality
Control	4	30	5.5	18.1	26	13
SS diet	12	94	5.3	2.2	0	100
SS " + liver	5	40	5.1	4.1	0	100
SS " + alfalfa	4	32	5.8	15.2	23	28

TABLE II.
Survival of Newborn Rats from Mothers on a High Pteroylglutamic Acid Diet.

Diet	No. of litters	No. of newborn	Birth wt, g	% of newborn surviving 20 days
Control	6	42	5.1	97
SS diet	8	56	5.2	91
SS " + alfalfa	8	55	5.2	96

sulfathiazole intragastrically, or intraperitoneally, at a level estimated to be about 10 times that ingested in the milk was without effect on the newborn rats from mothers on a normal diet. When small amounts of sulfathiazole (0.08%) were included in the diet, there was no effect on lactation. Since this dietary level of sulfathiazole produced about the same level of blood sulfathiazole as did 1% SS, the lactational failure could not be ascribed to a direct toxicity of SS or sulfathiazole.

A further experiment indicated that the fatality among the newborn rats from mothers receiving the diet containing SS was most probably the result of inanition. When newborn rats (12-36 hours after birth) from mothers on a normal diet were substituted for the newborn litter of a mother on a diet containing 1% SS, the normal newborn died within a few days. On the other hand, the newborn from mothers on the SS diet were successfully raised to weaning by mothers receiving a normal diet.

Since a toxic factor did not appear to be responsible, it was considered that the failure in lactation might be due to the need for some essential nutrient, normally synthesized by the intestinal flora, but now lacking because of bacterial suppression by the SS.

Experimental. In all the following experiments, female rats were placed on the control or experimental diets 10 days before mating. The females were kept on their respective diets until 20 days after the birth of their young. In the first series of experiments (Table I), the litters were limited to 8 young; in the second (Table II), to 7 young.

The control diet consisted of 28 g vitamin-free casein (SMACo), 56 g carbohydrate (sucrose or cerelose), 10 g lard, 2 g corn oil, 4 g salt mixture,[2] 1.0 mg thiamine HCl, 1.5 mg riboflavin, 1.0 mg pyridoxine HCl, 10 mg nicotinic acid amide, 4.0 mg calcium pantothenate, 100 mg inositol, 100 mg choline chloride, 100 mg p. aminobenzoic acid, 0.5 mg 2-methyl naphthoquinone, 0.05 mg biotin, 0.02 mg pteroylglutamic acid,[†] 2.0 mg α-tocopherol, 2500 IU vitamin A, and 360 IU vitamin D. The experimental diet (SS diet) was the same except that 1 g of succinyl sulfathiazole was substituted for an equal amount of carbohydrate. The diets were fed *ad libitum.*

A comparison was made of the effect of the control and SS diets on lactation. The survival time of the newborn rats was taken as a measure of lactation. Although some of the litters of newborn from the SS diet group had milk in their stomachs the first and second days, they were all dead by the fifth day

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[1] Williamson, M. B., Abstr., *Div. Biol. Chem., Am. Chem. Soc.*, 1948, 55C.

[2] Hubbel, R., Mendel, L., and Wakeman, D., *J. Nutrition*, 1937, **14**, 237.

[†] The pteroylglutamic acid was generously supplied by Dr. M. C. Lockhart of the Lederle Laboratories.

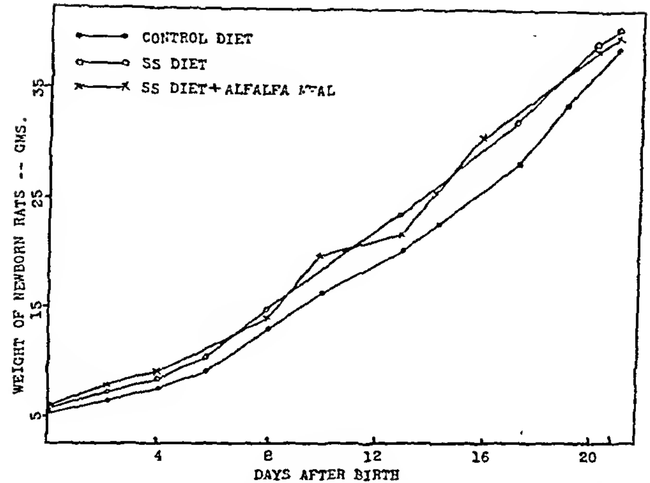

FIG. 1.

The rate of growth of newborn rats from mothers on a synthetic diet containing pteroylglutamic acid.

after birth; in no case was milk observed after the second day. This might be taken to indicate that in some cases, the crucial nutritional factor required to maintain lactation was not depleted until after one or two days of lactation. After the depletion, lactation failed with the consequent death of the newborn rats. Only 13% of the control newborn died before 20 days; it seems highly improbable that this mortality was due to lactational failure.

Further experiments in which supplements were added to the SS diet were undertaken. When 1% dry defatted liver powder was added to the SS diet, a slight but questionable increase in survival time was noted. In another experiment, 10% alfalfa meal was used. The casein content of the SS diet containing alfalfa meal was adjusted so that the total nitrogen content was the same as that of the control diet. The alfalfa meal supplement markedly improved the SS diet so that it was almost as efficient as the control diet in supporting lactation. The results of these experiments are shown in Table I.

Since it is known that added nutritional "stress" occurs during periods of pregnancy and lactation,[3] it was thought that there might possibly be some nutrient which was required at an even higher level than was supplied by the preceding diets. Richardson and Hogan[4] have reported the successful rearing of rat litters by mothers on a diet essentially the same as the control diet, except that it contained no pteroylglutamic acid or SS. The presence of SS in the diet, however, makes the necessity for exogenous pteroylglutamic acid crucial.[5] Therefore, the effect of a higher level of pteroylglutamic acid on lactation was investigated.

The control diet, the SS diet and the SS + alfalfa meal diet were supplemented to contain 1.0 mg of pteroylglutamic acid per 100 g of diet. Almost all the newborn rats from all groups survived until 20 days after birth. The results are indicated in Table II. The rates of growth of the newborn are shown in Fig. 1.

It can be seen that all the newborn grew at approximately the same rate, and hence, most likely received about the same amount of milk. It then follows that the level of pteroylglutamic acid in the maternal diet was at, or above, the optimal level for the support of lactation. Recent reports have shown the beneficial effects of pteroylglutamic acid on lactation.[6,7]

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[3] Ershoff, B. H., *Physiol. Rev.*, 1948, **28**, 107.

[4] Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep.*, *U.S.P.H.S.*, 1942, **57**, 1559.

[5] Richardson, L. R., and Hogan, A. G., *J. Nutrition*, 1946, **32**, 459.

It is interesting to note that the maternal requirement for pteroylglutamic acid appears to be very much greater during the period of lactation than during pregnancy. This is supported by the fact that the birth weights of all the newborn were found to be about the same (5.5 ± 0.8 g) regardless of the maternal intake of pteroylglutamic acid.

Summary. The inclusion of succinyl sulfathiazole in the diet of maternal rats during pregnancy depresses lactation. Relatively high levels of pteroylglutamic acid are required to maintain lactation. Higher levels of pteroylglutamic acid appear to be required during lactation than are required during pregnancy.

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[6] Nelson, M. M., and Evans, H. M., *Arch. Biochem.*, 1947, **13**, 265; 1948, **18**, 153.

[7] Sica, A. J., Allgeier, A. M., and Cerecedo, L. R., *Arch. Biochem.*, 1948, **18**, 119.

16921

Acetone-Ether Extracted Antigens for Complement Fixation with Certain Neurotropic Viruses.

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A method is described for the preparation of antigens for complement-fixation tests with certain neurotropic viruses. This antigen has been found to be practical, nonanticomplementary, specific and reliable. It is furthermore, easily prepared with ordinary laboratory equipment and can be made available, if necessary, within 6 hours from the time when the tissue is harvested, thus being useful for field work. Since the preparation depends on the elimination of the acetone-ether soluble fraction of the brain tissue with considerable reduction of lipid, it has the advantage of doing away with nonspecific reactions with Wassermann-positive human sera.[1,2]

Method of preparation. Brain tissue from mice infected intracerebrally with the desired virus is harvested; preliminary bleeding of the mice[2] is advisable in order to reduce the amount of hemoglobin present in the final product. The weighed tissue with 20 volumes of acetone is placed in a Waring blendor and allowed to run for 2 minutes. The blendor used for this purpose is surrounded by a metallic jacket which is filled with ice and water to maintain a low temperature; less preferably, the extraction can be carried out in the absence of this device. The suspension is then poured into a 250 ml bottle and centrifuged at 1500 rpm for 1 minute; the supernate is discarded. All the foregoing procedures should be rapidly performed. To the sediment is added 20 volumes (referred to the initial weight of the wet-brain tissue) of fresh acetone; the bottle is closed with a cork stopper, shaken by hand at intervals and kept at room temperature for 20 minutes. The preparation is then centrifuged as before, the supernate which is slightly opalescent, as are the subsequent supernates, is discarded. To the sediment is added 20 volumes of a mixture of equal parts of acetone and anhydrous ethyl ether. Again the suspension is kept at room temperature for 20 minutes, shaken occasionally by hand, centrifuged at 1500 rpm for 1 minute and the supernate discarded. The sediment is washed twice in succession with 20 volumes of ethyl ether each time and held for 20 minutes at room temperature. After the last ether extraction and centrifugation, the sediment is dried by connecting the centrifuge bottle with a vacuum oil pump; in a short time, from 15 minutes to 1 hour depending on the amount involved, the residual ether is evaporated and the sediment remains as a

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[1] DeBoer, C. J., and Cox, H. R., *J. Bact.*, 1946, **51**, 613; *J. Immunol.*, 1947, **55**, 193.

[2] Espana, C., and Hammon, W. D., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 101.

TABLE I.
Protein and Lipid Contents of Complement-fixing Antigens.

Antigen	Method of preparation	Diluent/original brain tissue	Protein, mg/ml	Lipid, mg/ml
Japanese B	Acetone-ether extraction	3:1	10.7	0.18
Normal brain		1:1	38.8	1.33
” ”		5:1	4.5	0.37
Japanese B	No extraction with acetone-ether; frozen and thawed	10:1	1.2	0.32
Normal brain		10:1	2.0	0.30

dry, fine powder. It is necessary to set a trap preferably containing cotton soaked in heavy oil between the bottle containing the material and the pump, owing to the fact that the evaporation proceeds rapidly and some of the fine powder may be drawn into the pump. After drying, the material always kept in the same bottle is resuspended in physiological saline solution; the amount of the latter can vary between 2 and 5 times the weight of the initial brain tissue, according to the concentration desired; ordinarily, 3 volumes of saline suffice. The suspension is placed in the refrigerator overnight; almost equally good results are, however, obtained if it is kept at room temperature for 30 minutes. The suspension is centrifuged in the angle-head centrifuge at 10,000 rpm for 1 hour; the supernate is pipetted off, Merthiolate in final concentration of 1:10,000 added and it is then stored in a glass-stoppered bottle at 2°C. This extract constitutes the antigen.

Complement-fixing antigens have been prepared by this means with the following viruses: Japanese B, St. Louis, Western equine, and Russian Far East encephalitis. No attempt has been made to lyophilize the preparations; in liquid form they can be kept at least 4 months at 2°C. It should be borne in mind that the antigens contain active virus immediately after preparation; it may be possible to inactivate it by formolization or ultraviolet radiation. The writer, however, employs the material without the inactivating treatment.

The general procedure for carrying out the complement-fixation test and for preparation of animal immune sera has been described elsewhere.[3] Unless otherwise indicated the acetone-ether antigens used in the following experiments had been prepared by resuspending the final material in 3 volumes of diluent, referred to the initial wet-brain tissue weight.

Protein and lipid content. Although the precise chemical nature of the antigens is unknown, it was observed that the titer of samples deriving from the same lot increased with the concentration of protein in solution. When lipids were not extracted, increased protein concentration in the antigen resulted in increased lipid in suspension, thus yielding anticomplementary and nonspecific effects. When antigens were prepared by the present method, the concentration of protein could be increased considerably, with resulting higher titer, and lipids, although not wholly removed, were present in such small amount as to give no anticomplementary reaction. Table I shows the result of determinations of protein and lipid in antigens prepared by the present, as well as in antigens prepared by a previously described method,[4] without lipid extraction.*

It can be seen that the ratio of protein to lipid which was 7 to 1 or less in the nonextracted antigens, was 60 to 1 in some of the acetone-ether treated antigens. Furthermore, the concentration of protein could be increased in the latter to a value far beyond that shown by the nonextracted antigens, with no indication of anticomplementary effect.

Wassermann-positive sera. An investigation of the possible nonspecific reaction with Wassermann-positive human sera† revealed that under present conditions of inactivation

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[3] Casals, J., *J. Immunol.*, 1947, **56**, 337.

[4] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

* Protein was estimated by quantitative biuret test. Lipid determinations were made by Dr. E. H. Ahrens, Jr., of the Rockefeller Institute Hospital, to whom many thanks are due.

TABLE II. *Complement-fixation Reaction with Wassermann-positive Human Sera Inactivated at 63°C for 3 Minutes and Acetone-ether Extracted, and Nonextracted Antigens.*

	Acetone-ether extracted antigen								Nonextracted, frozen and thawed antigen										No antigen, saline	
	West. equine				Japanese B				Japanese B					St. Louis						
Serum dilutions Serum No.	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32	1:2	1:4
1	0	0	0	0	0	0	0	0	4	4	3	±	0						0	0
2	0	0	0	0	0	0	0	0	3	2	1	0	0						0	0
3	0	0	0	0	0	0	0	0	2	1	0	0	0						0	0
4	2	0	0	0	3	2	0	0	4	4	3	±	0						0	0
5	0	0	0	0	0	0	0	0	4	3	±	0	0						0	0
6	0	0	0	0	0	0	0	0	3	3	3	±	0						0	0
7	0	0	0	0	0	0	0	0						0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0						4	4	0	0	0	0	0
9	0	0	0	0	0	0	0	0						0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0						3	3	±	0	0	0	0
11	0	0	0	0	0	0	0	0						2	±	0	0	0	0	0
12	2	0	0	0	2	0	0	0						4	4	1	±	0	2	0

Degree of fixation is expressed from 0 indicating no fixation to 4 indicating complete fixation.

(63°C for 3 minutes) they reacted nonspecifically with antigens of the former type, but not generally with those acetone-ether treated (Table II).

Anticomplementary effect. Numerous titrations of the anticomplementary effect of acetone-ether extracted antigens have been carried out under test conditions, namely incubation of the mixture of antigen and complement for 18 hours at 2-4°C. Table III shows the result of the titrations of complement in 3 different tests, in the presence of extracted antigens and of saline. When the antigens were prepared by resuspending the extracted brain tissue in 2 or more volumes of diluent (referred to the initial brain weight), the titer of complement was the same whether antigen was present or not. The concentrations of protein and lipid in these antigens were of the order of 20 mg or less, and 0.6 mg or less, per ml, respectively. On the other hand, when 1 volume of diluent or less was used, the resulting antigens were somewhat anticomplementary and gave a degree of nonspecific cross-reaction.

Specificity of the Reaction. The specificity of the complement-fixation reaction with acetone-ether extracted antigens was investigated mainly with mouse hyperimmune sera, and in a few cases, with human convalescent sera. Serial twofold dilutions of serum beginning with dilution 1:2 were tested against several antigens, either undiluted or diluted as indicated. The high dilutions of serum served to indicate that high titers of antibody could be obtained with these antigens, whereas the low dilutions showed that no cross-reactions occurred between different viruses, except in those cases when they are known to exist naturally, as for instance, between Japanese and St. Louis encephalitis viruses.[5] Examples of such tests are given in Table IV, in which are noted the high titers and the specificity of the antigens.

Titer of Antigens. When the extracted

[†] Wassermann-positive sera were obtained through the courtesy of Miss J. Haber, New York Hospital, New York. When received these sera had already been inactivated by heating at 63°C for 3 minutes.

[5] Casals, J., *J. Exp. Med.*, 1944, 79, 341.

TABLE III.
Titration of Anticomplementary Power of Acetone-ether Extracted Antigens.

Test No.	Antigen	Diluent/original brain tissue	Protein, mg/ml	Complement: diluted fresh guinea pig serum 1:30 Amount of complement in ml .16	.14	.12	.10	.08	.07	.06	.05	.04
1	None, saline			0	0	0	0	0	0	±	2	3
	Normal brain	2:1	16.0	0	0	0	0	0	0	±	2	3
	Japanese B	1:1	30.0	0	0	0	0	±	2	2	3	4
	West. equine	3:1	9.0	0	0	0	0	0	0	1	2	2
2	None, saline				0	0	0	0	±	1	2	
	Normal brain	3:1	10.9		0	0	0	0	0	1	2	
	Japanese B	3:1	11.7		0	0	0	0	±	1	3	
3	None, saline			0	0	0	0	1	2	2		
	Normal brain	0.9:1	45.0	0	0	2	3	4	4	4		
	Japanese B	3:1	11.7	0	0	0	0	1	2	3		

TABLE IV.
Complement-fixation Tests with Acetone-ether Extracted Antigens; Mouse Hyperimmune and Human Convalescent Sera.

Antigen			Serum			
Type	Protein, mg/ml	Dil.	Species	Japanese B	Russian Far East	Western equine
Japanese B	9.7	1:4	Mouse	1:128*	0†	0
Russian Far East	8.5	1:4		0	1:64	0
West. equine	9.0	1:4		0	0	1:8
Japanese B	30.6	Undil.	Mouse	1:32		0
West. equine	7.9	″		0		1:8
Normal brain	17.5	″		0		0
Japanese B	9.7	″	Human, A‡	1:512		
			″ B	1:256		
West. equine	9.0	″	″ A	0		
			″ B	0		

* *Highest dilution* of serum giving a 2+, or better, reaction. The first tube had in all cases serum dilution 1:2.

† O indicates that no fixation occurred in any tube.

‡ The 2 human sera in this test were derived from individuals convalescent from Japanese B encephalitis.

brain tissue was resuspended in 3 volumes of diluent, the titer of the antigens was between 1:16 and 1:128, depending on several factors, such as the type of virus, the particular lot of brain tissue, and the relative amounts of serum and antigen that were employed. With respect to the latter it should be noted that, unlike what happens with nonextracted antigens,[3] the titers of both serum and antigen depend on their dilutions; more so does this apply to serum of which the titer is closely correlated with the number of units of antigen (Table V). This observation confirms that of Espana and Hammon,[2] who observed a similar phenomenon with their benzene-extracted antigens. Thus if a "box" titration were performed, with dilutions of serum tested against dilutions of antigen, results as given in Table V could, as a rule, be found.

It can be seen in Table V that the titer of the serum varied from 1:32 to 1:128 depending on whether antigen was used undiluted or in dilution 1:16. Conversely, the titer of the antigen was 1:64 when serum was used in dilution 1:2 and 1:128 with serum in dilution 1:16. In order to secure the highest titer for a given serum it seems advisable to use, as recommended by Espana and Hammon,[2] from 8 to 16 units of antigen. One unit of antigen is the amount contained

TABLE V.
Complement fixation Test with Japanese B Encephalitis Virus Antigen and Mouse Hyperimmune Serum. Acetone-ether Extracted Antigens.

Antigen		Dilutions of serum							
Type	Dil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Japanese B	1:1	4	4	4	4	3	0	0	0
	1:2	4	4	4	4	4	0	0	0
	1:4	4	4	4	4	4	2	0	0
	1:8	4	4	4	4	4	4	±	0
	1:16	4	4	4	4	4	4	2	0
	1:32	4	4	4	4	4	4	2	0
	1:64	3	3	4	4	4	3	1	0
	1:128	0	±	1	2	1	±	0	0
	1:256	0	0	0	0	0	0	0	0
West. equine	1:1	0	0						

in the highest dilution of antigen which in a box titration gives a 2+ or better reaction; in the example shown, dilution of antigen 1:128 equals one unit.

To conclude, reliable, high-titered antigens for complement-fixation tests with certain neurotropic viruses have been prepared by a simple method of extraction at room temperature of infected brain tissue with acetone and ethyl ether.

16922

Intravenous Infusions of a Combined Fat Emulsion into Human Subjects.*

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In previous papers from this laboratory, an emulsion of fat combined with protein and glucose was reported to have been administered intravenously into dogs with safety and with nutritional benefits.[1,2] The overall advantages of this intravenous emulsion were such that it warranted the present clinical investigation of its effects on human subjects. This however was not done without precedent for Holt had pioneered studies on intravenous fat in a series of pediatric patients whilst others reported sporadic attempts in adult humans.[3,4] The present report is devoted to the method of preparation of the combined fat emulsion and the effects of its intravenous infusion on human subjects.

Preparation of the Combined Fat Emulsion. The successful preparation of this emulsion is dependent upon adequate and thorough homogenization and emulsification which is accomplished in a specially constructed homogenizer adapted to medical usage. Basically the mechanism is that of a high pressure pump which forces the fluid mixture through a minute orifice against the resistance of a strong steel springed valve. The machine consists of three component parts: a reservoir tank with a high speed agitator incorporated

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* Aided by grants from the Ben Lewis Fund in Experimental Surgery. Clinical investigation done at Goldwater Memorial Hospital (New York University, Third Surgical Division).

[1] Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.

[2] Shafiroff, B. G. P., Baron, H. C., and Roth, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 387.

[3] Holt, E., Jr., Tidwell, H. C., and Scott, T. F. McNair, *J. Pediat.*, 1936, **6**, 151.

[4] Clark, D. E., and Brunschwig, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 329.

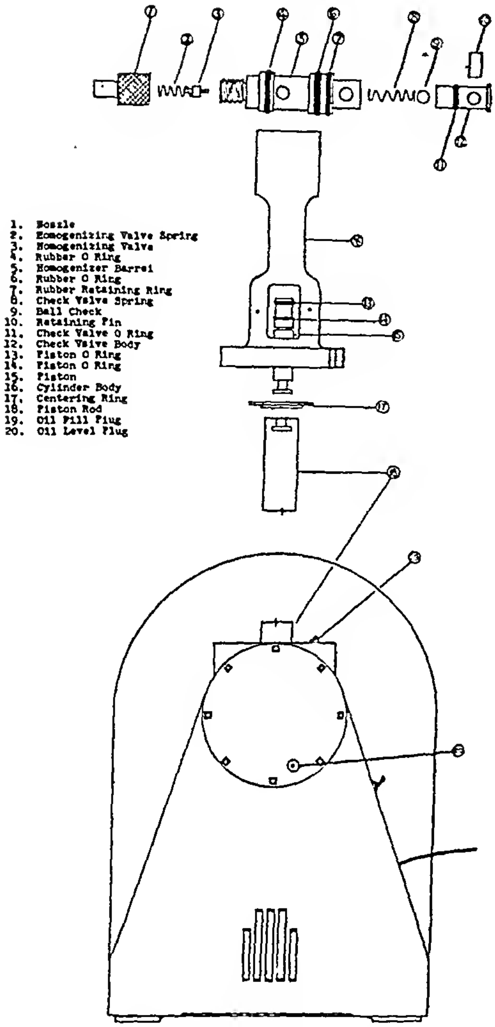
1. Nozzle
2. Homogenizing Valve Spring
3. Homogenizing Valve
4. Rubber O Ring
5. Homogenizer Barrel
6. Rubber O Ring
7. Rubber Retaining Ring
8. Check Valve Spring
9. Ball Check
10. Retaining Pin
11. Check Valve O Ring
12. Check Valve Body
13. Piston O Ring
14. Piston O Ring
15. Piston
16. Cylinder Body
17. Centering Ring
18. Piston Rod
19. Oil Fill Plug
20. Oil Level Plug

FIG. 1.
Diagrammatic sketch of homogenizer apparatus.

into its cover, a motor and belt mechanism to drive the compressor piston and a homogenizer block fitted with a system of inlet and outlet valves. (See Fig. 1 for diagram of the apparatus.) Within the block a high internal pressure is created which is expended on the homogenization of the mixture. The emulsion is prepared entirely under sterile conditions. The homogenizing block and all the parts in contact with the mixture can be detached as a unit and autoclaved before each batch of homogenate is processed. The ingredients of the mixture already in sterile form are drained from their respective containers into the reservoir tank through a special opening in the cover provided for the purpose. The mixture is converted into a blend or crude emulsion after a 5-minute period of high speed agitation. The blend is then allowed to flow into the homogenizing chamber within which the emulsifying process is completed. The emulsion is continuously recyclized through the block under 2500 pounds pressure for a period of 20 minutes and then collected in sterile liter flasks which are capped and stored ready for intravenous use. Test samples of the homogenate of each batch are examined for particle size and for sterility.

The constituents of the emulsion are solutions of protein hydrolysate (amigen, 10%), 50% glucose, intravenous gelatin (Knox P-20, 6%), and refined coconut oil. All except the latter are available as sterile nonpyrogenic solutions in sealed flasks. The coconut oil is autoclaved before its addition into the tank. The combined fat emulsion used in the present studies is an homogenate of approximately 10% fatty oil, 5% protein hydrolysate, 5% glucose, and 2% gelatin. The caloric value of the emulsion is about 1,300 calories per liter. The pH of the emulsion is about 6.2. The particle size of the emulsion is less than one micron and exhibits active Brownian movement on microscopic examination.

In the present studies intravenous gelatin was used exclusively as the emulsifying agent. After many experiments it was found to be superior to the soy bean phosphatides, lecithin, and a variety of artificial stabilizing agents. Intravenous gelatin *per se* has been proved to be non-toxic and non-allergenic. As an emulsifying agent a relatively small amount, less than 3%, is required for the emulsification of 10% fatty oil. The stability of the 10% combined fat emulsion has been observed for a period of more than two years without "creaming" or "oiling out."

Method of Study. The combined fat emulsion was administered without selectivity to hospital patients who were being treated for a variety of surgical conditions. Many of these patients ran a chronic low grade fever. The studies were started in the morning with

TABLE I.
Immediate Effects from Infusion of One Liter of 10% Combined Fat Emulsion.

Temperature reactions		Constitutional reactions			
Elevation	%	Subjective	%	Objective	%
< 1°	29	Nausea	1	Chills	9
1° to 1.9°	50	Headache	3	Vomiting	2
2° to 2.9°	13	Dizziness	1	Cough	2
> 3°	8	Fatty taste	1	Allergic	1

the patient in the post-absorptive state after a fasting period of 14 hours. Before the infusion was started a sample of blood was taken, the morning urine was obtained, and temperature, pulse, respirations, and blood pressure were noted. The infusion was then given intravenously at a specific rate ranging from 20 to 80 drops per minute varying in different patients. During the course of the infusion and throughout the post-infusion phase until the next morning blood and urine samples were taken and the above mentioned clinical notations were made at regular intervals. The following tests were made routinely on the blood: hemoglobin, red cell count, white cell count with differential, sedimentation time, chylomicron count, and specific gravity. In special cases tests were made for the quantitation of blood lipids with the refractometer and the nephelometer, for blood volume by the Evans blue method and for blood viscosity with the Hess viscosimeter. Clinically, subjective and objective constitutional reactions relative to the intravenous infusion of the emulsion were studied. X-rays of the lungs were taken in series for any lesions due to fatty infiltration, pulmonary irritation or pulmonary emboli.[5] Final check examinations were made 2 weeks after completion of the infusions for hemolytic anemia and for possible liver or kidney damage. Surgical liver biopsies were obtained from several patients who received the emulsion. These specimens were prepared for microscopic analysis by staining with osmic acid, oil-red -O, Sudan IV, and hematoxylin-eosin.

Results. The combined fat emulsion was infused intravenously into 76 human subjects for a total of more than 250 liters. An average of 4 liters of emulsion was administered to each of 35 patients. One patient received a total of 16 liters of the emulsion which was the maximum amount infused into any one patient. Five patients received 2 liters of the emulsion during one infusion period instead of a single liter.

Temperature changes during and immediately after the infusion were computed according to the degree of elevation above the pre-infusion temperature. The results have been classified in Table I on a percentage basis from the protocols of the last 200 infusions. These values were not corrected for temperature changes due to the disease of the patient. Constitutional reactions, subjective and objective, have also been outlined in Table I. The incidence of chills averaged 9% of all infusions, the majority of which came concomitantly with a high temperature reaction. In 2% chills occurred independently, without any temperature elevation. The chill reaction was the severest type of reaction encountered in the present series. Allergenic reactions were usually of the urticarial type due to sensitivity to the amigen component in the emulsion. The latter reaction could be obtained by the infusion of amigen alone and was found to be controllable by antihistaminic drugs given in conjunction with the administration of the emulsion. Cough and vomiting were moderately severe reactions which were usually controlled by reduction of the speed of intravenous administration of the emulsion.

Changes of blood pressure in response to the injection of the emulsion were variable. During the infusion period there was generally a slight elevation of blood pressure followed by a moderate fall in pressure which persisted for 2 hours after completion of the

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[5] Jirka, F., and Scuderi, C. S., *Arch. Surg.*, 1936, 33, 708.

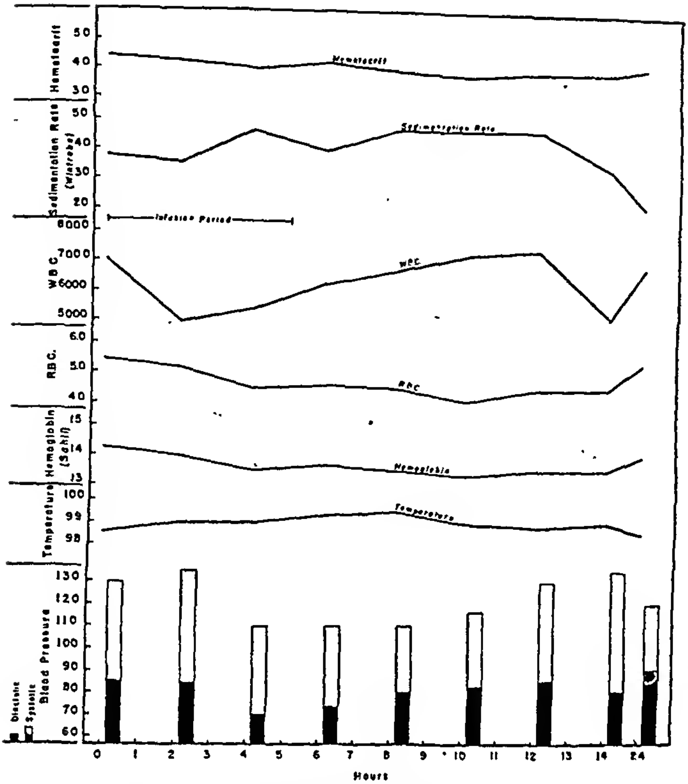

FIG. 2.

Graphs showing the effects of the intravenous infusion of one liter of the 10 per cent combined fat emulsion. The values of the individual curves were averaged from all the protocols.

infusion. The elevation of systolic pressure did not exceed 20 mm Hg and the fall in systolic pressure ranged from 10 to 25 mm Hg. The diastolic pressure tended to fall towards the end of the infusion but also returned to its normal level (Fig. 2).

The values for hemoglobin, hematocrit cell volume and red and white cells tended to fall below their initial values during the infusion phase of the emulsion and returned to their original levels shortly before or after the termination of the infusion. The erythrocyte sedimentation rate showed an increase in rapidity of sedimentation which averaged 35% greater than the pre-infusion sedimentation time, and also returned to its initial level. The differential white count was not indicative of any trend. Leukopenia was found to occur in 2% of infusions concomitant with chill and temperature reactions (Fig. 2 and Table II).

Counts of chylomicrons were made from peripheral venous blood under dark field illumination with a net micrometer in the eyepiece of the microscope. The curve of chylomicron counts beginning from the postabsorptive level through the infusion phase of the emulsion was characteristic. In the postabsorptive state the initial pre-infusion count varied from zero to 10 chylomicrons. After the infusion was started there was a rapid rise in the number of chylomicrons to a peak. At about the mid-point of the infusion the chylomicrons began to decrease in number and return slowly to the initial level. Fig. 3 was drawn from the average values of all the chylomicron counts. The chylomicron curve was found to be modifiable by the speed of infusion, the amount of fat emulsion administered and the oral ingestion of food.

Quantitative microchemical determinations of blood lipids showed a rapid rise of blood fat

TABLE II.
Effects of Intravenous Infusion of One Liter of Combined Fat Emulsion Into a Subject.

Time (min.)	Amt infused (ml)	Temperature Fahrenheit (degrees)	Pulse rate (per min.)	Blood pressure (mm Hg)	Hemoglobin Sahli (g)	Sedimentation time (mm/hr)	R.B.C. count (million)	W.B.C. count	Hematocrit (%)	Plasma refractive index	Chylomicra count ¼ unit	Blood lipids (mg)	Urine acetone
0	—	98.0	80	132/98	14.0	24	4.75	7000	44	1.350	8	800	neg.
60	240	97.8	80										
120	350	98.0	98	140/100	13.0	23	4.75	6800	41	1.349	350	1070	neg.
180	520	98.6	100										
240	625	98.6	96	140/110	13.0	36	4.70	5000	40	1.349	150	905	1+
300	—	98.8	104										
360	1000	98.6	104	140/110	13.2	36	4.75	5600	41	1.351	250	625	1+
420	—	98.2	100										
480	—	99.0	104	130/110	13.4	21	4.90	5800	42	1.350	150	810	neg.
540	—	98.0	104										
600	—	99.0	100	150/110	13.0	36	4.75	6000	41	1.350	100	705	neg.
660	—	99.0	98										
720	—	99.2	100	150/110	13.2	36	4.90	6600	42	1.350	50	770	neg.
24 hr	—	—	—	—	—	28	5.20	6800	43	—	10	685	neg.

and an equally rapid fall to normal limits within 2 hours after completion of the infusion. Nephelometric examinations of plasma samples showed variations in the degree of turbidity comparable to the variations obtained by microchemical analysis and chylomicron counts made on the same samples. Viscosimetric determinations and refractometric analysis showed changes in the blood during the course of the infusion which were consistent with the changes already noted. The specific gravity of the plasma showed a temporary dilution effect due to the infusion followed by a return to normal limits.

Consecutive urine studies were made in 43 infusion tests. In no case was a positive test for blood or urobilinogen obtained which could be attributed to the emulsion. Chemical tests for fat in the urine were negative. Dark field examination of the urine for chylomicrons sometimes showed a moderate elevation in the number of fat particles although the chemical test for fat was negative. A positive reaction was obtained occasionally for acetone varying from 1 + to 2 + in 2 or 3 of the intermediate urine specimens taken during the course of the infusion of the emulsion. When the infusion was completed the positive tests for acetone became negative. A positive test for diacetic acid was found rarely.

Hematologic follow-up studies in patients who received either single or multiple infusions showed no signs of hemolytic anemia. Likewise x-rays of the lungs gave no evidence of fatty emboli or pulmonary irritation. The photographs in Fig. 4 and Fig. 5 were taken to show the microscopic appearance of human liver sections. The first, Fig. 4, was taken a few hours after an infusion and the second, Fig. 5, 36 hours after the last of 9 consecutive infusions of the combined fat emulsion. Liver and kidney function studies showed no pathologic physiology ascribable to the use of the emulsion.

In 5 patients it was necessary to discontinue the infusion of the emulsion before half of the contents of one liter had entered the vein. This was necessary because of the severity of the chill experienced by the patients. All of the 5 patients were in advanced states of malnutrition with severe anemia and with markedly diminished circulating blood volumes. Four of these patients were able to tolerate large volumes of the combined fat emulsion after the anemia and the blood volume were corrected by multiple blood transfusions.

Comments. The combined fat emulsion was administered intravenously to human patients with safety. The immediate and late effects of single and repeated infusions of the

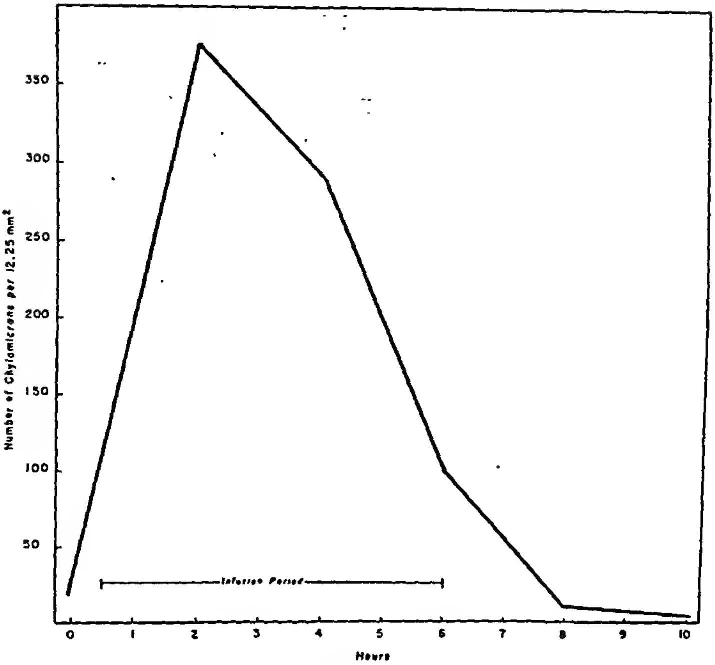

FIG. 3.

Graph of the curve of the chylomicron counts obtained during the infusion and post-infusion periods of the 10 per cent combined fat emulsion. The values on this curve have been averaged from all the chylomicron counts in the protocols.

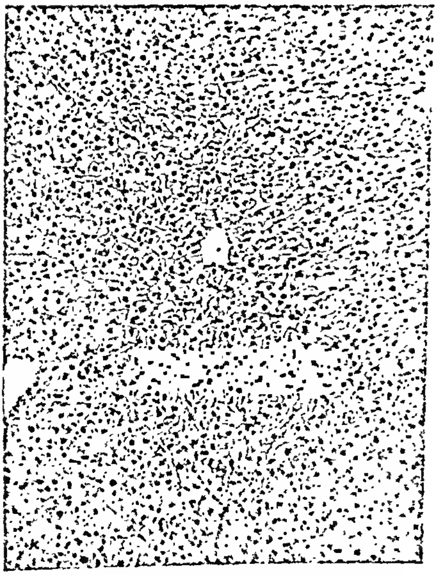

FIG. 4.

Low power magnification of human liver section focused on central vein. Surgical biopsy several hours after patient had received 2 liters of the 10 per cent combined fat emulsion. (H & E stain ×50).

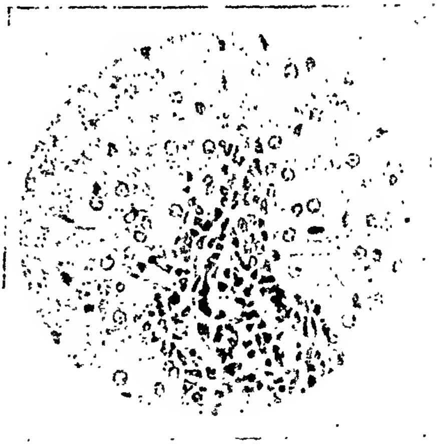

FIG. 5.

High power magnification of human liver section focused on periportal area. (H & E stain ×220). Surgical biopsy 36 hours after patient had received a total of 9 infusions of the combined fat emulsion.

emulsion were studied both from the clinical and the hematologic standpoint. A moderate elevation in temperature as a consequence of injection of the combined fat emulsion can be accounted for on the basis of an increase in total heat production due to the metabolism of fat plethora. The increased rate of blood sedimentation was believed to be associated with erythrocyte pseudo-agglutination caused by the infused gelatin. The variations in hemoglobin, etc., were related to the dilution effect of the emulsion. The incidence of constitutional reactions compared favorably with reactions encountered after blood transfusions. The sharp rise in the number of chylomicrons followed by the slower fall was indicative of the rate of disappearance of the intravenously injected fat from the blood stream. The appearance of acetone found in an occasional specimen of urine during the infusion phase may be explained on the basis of incomplete oxidation of the fat. In the follow-up we were unable to find any serious toxic effects or late sequelae ascribable to the emulsion.

Summary. The method of preparation of a 10% combined fat emulsion used in the present investigation was described. A combined fat emulsion with a potency of approximately 1300 calories per liter was infused intravenously into 76 human subjects. A variety of laboratory tests and clinical observations provided evidence in favor of its suitability as an intravenous emulsion.

The authors are indebted to the E. F. Drew. Co., to the Mead Johnson Co., and to the C. F. Knox Co., for generous supplies of material used for the preparation of the emulsion. The homogenizer was made by the C. W. Logeman Co., New York City.

16923

Influence of Temperature on the Distensibility of the Pubic Ligament.*

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The studies of Hisaw and his collaborators[1,2] have demonstrated that hormonal changes can profoundly influence the distensibility of an apparently physiologically inert tissue such as the pubic membrane within a relatively short time. In the present study we have attempted to determine the relative influence of changes in the physical environment, *i.e.* load and temperature, on the response of the pubic ligament of the guinea pig.

The experiments to be described subsequently, were performed in both intact preparations of non-gravid and in isolated preparations of gravid and non-gravid guinea pigs. In the former series hormonal influences were controlled by ovariectomy and injection of serum from pregnant rabbits. It was soon found that under our experimental conditions such hormonal factors played a lesser part than did slight variations in the environment, as *e.g.* caused by variations in the distance of a source of heat to the preparation.

The method used in studying the effect of temperature on the stretching of the pubic ligament in the intact animal is illustrated in Fig. 1. The animal was kept under deep nembutal narcosis during the entire experiment. Stretch on the pubic ligament was exerted by a weight which pulled a string loop fastened with one end to the pubis, at the other to the ischium. The other half of the pelvis was similarly fastened with a short loop of string to a fixed point. Recording took place with a third loop, fastened to the side on which the weight pulled; this arrange-

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* This investigation was carried out with the aid of a grant from the Harlan Shoemaker Fund for Paralytics, Los Angeles, Calif.

[1] Hisaw, F. L., *Anat. Rec.*, 1927, **37**, 126.

[2] Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmadge, R. V. N., Kleinholz, L. H., and Hisaw, F. L., *Endocrinol.*, 1944, **34**, 103.

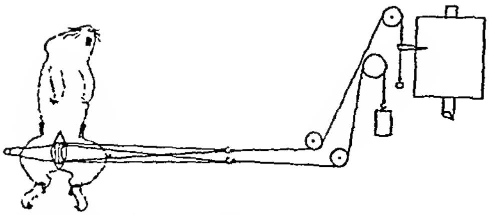
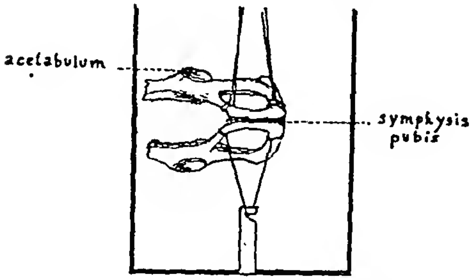


FIG. 1.
Diagram of the arrangement of stretching the pubic ligament of a guinea pig *in vivo*.



• FIG. 2.
Part of the arrangement for the experiments *in vitro*, showing the attachment of the strings to the preparation.

ment made it possible to record more nearly the actual change in the pubic membrane only, without interference of the stretching of the wire by the stretching weight. The animal was held in position between two nails driven just above the hindlegs into the board on which the preparation was mounted and by staples which held the hind legs fastened to the board.

A similar technic was used for the recording of the stretch of the isolated preparation. In this case the pelvis was cut just above the acetabulea. The short single loop on one side was fastened to a hook in the bottom of a cylindrical container, made of brass, which could be submerged in a constant temperature bath. The preparation itself was bathed in mammalian Ringer solution (Fig. 2).

Stretching was recorded on a kymograph and the preparations were subjected to stretch for up to 6 hours in the case of stretching *in situ*, whereas isolated preparations were kept for 12 hours or more. The number of animals in which the ligament was stretched *in vivo* and significant temperature changes were made, was 12. A far larger number, in which the temperature was kept more constant, and in which hormonal factors were varied, served to show that the variations observed with temperature change did not occur without such change. Eight ligaments were stretched *in vitro* under thermostatic conditions.

Results. Table I represents the results obtained when isolated pubic ligaments with the adjacent symphyses, were subjected to a constant load of 100 g at various temperatures for 12 hours. It will be seen that there is no noticeable difference between stretching at 25°C and 30°C. With higher temperatures the rate of stretching increases, and at 38°C is many times that at 25°C. Fig. 3 and 4 represent changes in the rate of stretching within a given preparation when temperature is varied. The stretch at the onset of the experiment is always larger at the same temperature than later, dropping exponentially. By changing temperature this curve can be greatly influenced. Thus from Fig. 3 it is seen that lowering the temperature from 37°C to 34°C almost completely prevents distension from a load of 100 g, but that stretching resumes when the original temperature is reestablished. This experiment was performed on a non-pregnant animal, with the preparation *in situ*. The temperatures measured are those of the Ringer fluid which surrounds the membrane. This fluid also prevents drying of the membrane. The experiment shown is representative of many performed in this way. From these it follows that a greatly increased rate of stretching is caused by a temperature rise between 33 and 36°C. Below this range in the non-pregnant guinea pig there is very little stretching with a load of 100 g, whereas above this range (up to 42°C) the rate further increases, but not so much.

Fig. 4 represents the stretching *in vitro* of

TABLE I.
Change in Distances of Isolated Pubic Membranes After 12 Hours of Stretching by 100 g virgin guinea pigs.

Temp.°C	Stretch in mm*	mm per hr
25	20	1.67
30	20	1.67
35	68	5.68
38	138	11.5

* As recorded on drum; about 4 times actual.

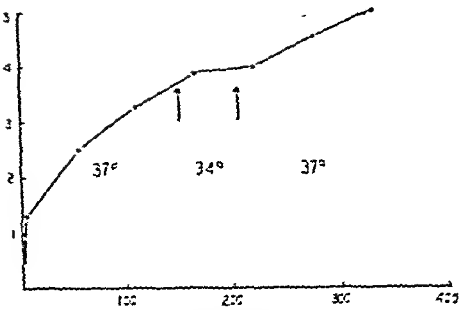


FIG. 3.

Plot of the increase in distance of the membrane, subjected to a stretch of 100 g *in vivo*, of a virgin guinea pig. weight 500 g. Between arrows temperature lowered from 37 to 34 degrees. Length in arbitrary units, each of which corresponds to about ½ mm. Time in units of 100 min.

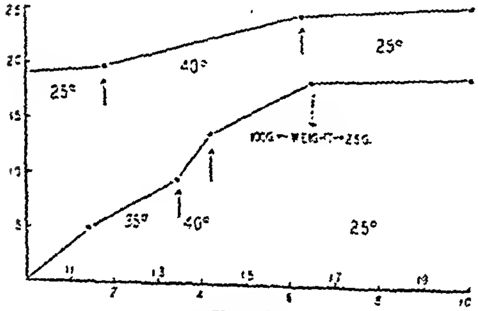


FIG. 4.

Stretching curve obtained from ligament of a pregnant animal *in vitro*, showing the influence of temperature and weight. Notice the much greater stretch than in Fig. 3. Upper curve is continuation of lower curve. Time in hr.

a ligament from an animal almost at term, to which 100 g stretching weight was applied. There is an increase in rate of stretch by changing from 35 to 40°C. In this pregnant animal lowering to 25°C does not prevent a very noticeable stretching. Even decreasing the stretching load to 25 g only reduces the stretching at 25°C to a value comparable to that found in non-pregnant pigs at body temperature. At 40°C the amount of stretching with 25 g load is again considerably increased.

This much greater sensitivity of the membrane of the pregnant animal is completely in accord with the view that the pressures present in the pelvis will be sufficient to push the bones apart. It is difficult to judge, how much pressure might occur under non-pregnant as compared to under pregnant conditions, the much smaller distensibility obviously prevents in most animals any great separation of the bones. Another factor which must be considered in these experiments is the presence of ligaments joining the pelvic girdle to the spinal column. These do possibly participate in the experiments on the membrane *in situ*, but not in the case of the isolated preparations. This may be the main reason, why the stretching curves of the latter were always considerably freer from small irregularities than the former.

It may be pointed out that the great influence of temperature changes between 33 and 37°C on the stretching of the pubic ligament raises interesting questions concerning the behavior of other ligaments in the body, which, by their location might be subject to temperature changes in this range under normal living conditions.

Summary. The effect of temperature on the distensibility of the pubic ligament of the guinea pig was investigated in intact and isolated preparations using a constant load. Both types of preparations exhibited a marked dependence of stretching on temperature between 30 to 40°C with the mode lying in the range 34-35°C under conditions of moderate loading.

Distension at 30°C is negligible, while at temperatures above 34 it is rapid. Preparations from pregnant animals show a lower threshold both for temperature and weight.

The authors want to thank Dr. D. R. Howton and Mr. H. H. Crawford for their assistance during the earlier part of this investigation.

16924

Degranulation of Beta Cells of Rat's Pancreas by Glucose Correlated with Alterations in Glucose Tolerance.[*]

CARL A. PETERSON. (Introduced by B. J. Clawson.)

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After the relationship of the pancreas to diabetes had been established by Von Mering and Minkowski,[1] and before the discovery of insulin, Allen[2] and Homans,[3] working independently, demonstrated changes in the beta cells of the islands of Langerhans in partially depancreatized diabetic dogs. Allen postulated that these observed alterations would be reversible, and Copp and Barclay,[4] after the discovery of insulin, proved this hypothesis to be correct by observing the recovery of the beta cells in partially depancreatized diabetic dogs treated with insulin. Allen explained this phenomenon by postulating that the beta cells were the source of the antidiabetic hormone and that an excessive functional demand caused an exhaustion of these cells and produced the histological changes he observed—degranulation and vacuolization. These early observations, verified and amplified by later research, were made using the Lane Bensley technic for staining the beta cells. If one can judge from the photomicrographs included in these early reports this method gave very inadequate results and the staining would not compare in quality or accuracy with the results produced by the Gomori stain[5] which was used exclusively in these experiments.

More recently diabetes has been produced experimentally by methods other than pancreatectomy; by the injection of the hormones of the anterior pituitary[6] and thyroid,[7] by the injection of alloxan,[8] and by the injection of large amounts of glucose.[9] And when a diabetic state has been established alterations in the beta cells of the pancreas, degranulation, vacuolization, and necrosis, are invariably found to be present.

In other experimental methods the changes *are limited to degranulation of the beta* cells and diabetes does not develop. Starving a white rat or feeding it an exclusively fat diet will completely degranulate the beta cells of the pancreas.[10] Repeated daily injections of protamine zinc insulin in the rat will produce degranulation of the beta cells.[11,12] Gomori[13] and associates demonstrated variable degranulation of the beta cells in the guinea pig following a single intraperitoneal injection of glucose.

The present investigation is concerned with the changes produced in the beta cells in the pancreas of the white rat and the alterations in the glucose tolerance following a single intra-cardiac injection of glucose.

Materials and methods. White rats of the Sprague-Dawley strain were used exclusively. Rats of various ages and weights and of both sexes were used. Glucose was given in 20%

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[*] This work has been done under a grant from the Office of Naval Research made to Dr. E. T. Bell and a Research Fellowship from the U. S. Public Health Service to Dr. Carl Peterson.

[1] von Mering, J., and Minkowski, O., *Zentralbl. f. Klin. Med.*, 1889, **10**, 393.

[2] Allen, F. M., *J. Metabolic Research*, 1922, **1**, 5.

[3] Homans, J., *J. Med. Research*, 1914, **30**, 49.

[4] Copp, E. F., and Barclay, A. J., *J. Metabolic Research*, 1923, **4**, 445.

[5] Gomori, G., *Am. J. Path.*, 1941, **17**, 395; Bell, E. T., *Am. J. Path.*, 1946, **22**, 631.

[6] Young, F. G., *Lancet*, 1937, **233**, 372.

[7] Houssay, B. A., *Clinical Proc.*, 1946, **5**, 223.

[8] Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

[9] Dohan, F. C., and Lukens, F. D. W., *Science*, 1947, **105**, 183.

[10] Barron, S. S., and Bell, E. T., *Arch. Path.*, in print.

[11] Peterson, C. A., unpublished work.

[12] Latta, J. S., and Harvey, H. T., *Anat. Rec.*, 1942, **82**, 281.

[13] Gomori, G., Friedman, N. B., and Caldwell, D. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 567.

TABLE I.
Results of One Injection of Glucose into Blood Stream of White Rat.

Rat No.	Sex	Wt, g	G glucose/kg	Hr inj. to autop.	Beta gran.	Rat No.	Sex	Wt, g	G glucose/kg	Time inj. to autop.	Beta gran.
20	M	200	0.25	1	3	307	M	300	3	5 min.	3
X	M	230	0.5	2	3	308	M	340	3	10 min.	1
E	F	130	1	¼	3	R	M	280	3	15 min.	0
D	F	160	1	½	3	Q	M	420	3	30 min.	0
C	M	120	1	¾	3	P.	F	310	3	45 min.	0
B	F	185	1	1	2	303	M	330	3	12 hr	0
A	M	150	1	1½	2	310	F	300	3	24 hr	0
K	M	220	2	1	1—	T	M	280	3	24 hr	1
L	F	190	3	1	0	U	M	400	3	48 hr	2
M	M	250	4	1	0	311	F	320	3	48 hr	3
N	M	210	6	1	0	V	M	400	3	72 hr	3
O*	M	360	3	1	0	Z*	F	230	3	1 hr	0

* 3 units of crystalline zinc insulin per 1.0 g glucose added to syringe.

solution in distilled water via needle and syringe directly into the left ventricle of the heart of the rat previously anesthetized with ether. Blood for glucose determinations was withdrawn by the same method and the Folin-Wu micro-method was used for the determinations. The rats were sacrificed at various intervals by injecting air into the left ventricle of the heart. All pancreases were fixed in Bouin's fixative and stained with Gomori's stain. A pancreas from a normal rat was included with each staining and all estimations of partial degranulation were made with reference to this control tissue. The degree of granulation was graded from 3, representing a pancreas with all the beta cells fully granulated, to 0, a totally degranulated pancreas. The designation 1 indicates a pancreas with very marked degranulation of the beta cells while 2 indicates a definite partial degranulation but not as marked as 1. The glucose tolerances were determined by injecting 0.25 g per kilo body weight of 20% glucose into the left ventricle of the heart.

Several experiments were attempted: Glucose in concentrations from 0.25 g per kilo body weight to 6.0 g per kilo was injected. Rats were then sacrificed at intervals from 5 minutes to several days. Glucose tolerances, blood glucose levels, and urine sugars were determined at varying intervals. All determinations of glycemia were made in the fasting state; all urine sugar recordings were determined on overnight specimens. In 2 rats 3 units of crystalline zinc insulin per 1.0 g of glucose were added to the syringe and injected simultaneously with the glucose. One rat was given 12 single injections of glucose, 3.0 g per kilo, over a period of 2 weeks.

Results. The beta cells of the islands of Langerhans were invariably totally degranulated within 15 minutes after the injection if 3.0 g per kilo, or higher concentrations of glucose were used. Lesser concentrations gave variable results: Less than 1.0 g per kilo resulted in no perceptible degranulation; 1.0 g per kilo reduced the granulation to the level of 2 in one hour; 2.0 g per kilo reduced the granulation to 1 or 1— at the end of one hour.

This process of degranulation was found to be reversible; regranulation of the beta cells followed degranulation. However, a delay of about 48 hours was observed. Twenty-four hours after the injection the granulation was found to remain markedly reduced (0-1); but in 48 hours the granulation appeared to be almost complete (2-3).

Glucose tolerance studies on rats 24 hours after the single injection of glucose (3.0 g per kilo) revealed curves of the diabetic or potential diabetic type (Fig. 1a). One week after the injection the glucose tolerance had returned to normal.

When 3 units of crystalline zinc insulin per 1.0 g of glucose were added to the syringe a marked reduction in the hyperglycemia followed, but not for approximately 30 minutes during which interval degranulation of the beta cells took place (Fig. 1b).

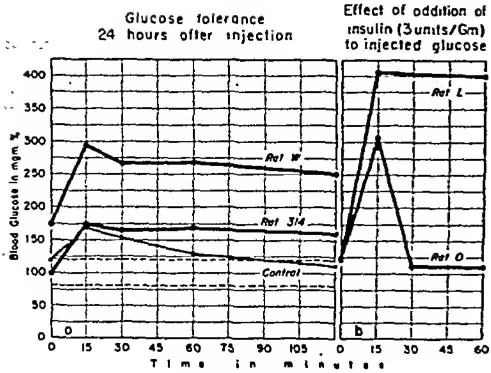

FIG. 1a.

Rats W and 314 received injection of glucose, 3.0 g/kg, 24 hr previous. The curves represent upper and lower extremes of glucose tolerance. Most other determinations fell in between.

FIG. 1b.

Rats L and O were both given glucose 3.0 g/kg. *Zinc crystalline insulin, 3 units per g of glucose*, was added to the syringe in injecting rat O.

Fasting blood sugars, determined 24 hours after the injection, were found to be elevated in almost every instance. One rat, No. 314, which showed a fasting blood sugar within the normal range, was found to have a glucose tolerance curve of a potential diabetic type (Fig. 1a). As would be expected most rats also showed a marked glycosuria 24 hours after the injection. This usually disappeared by 48 hours.

From these observations it became apparent that a correlation existed between the degree of beta cell granulation, the level of glycemia, the type of glucose tolerance curve, and the degree of glycosuria.

Attention is again directed to rat 314, glucose tolerance of which is illustrated in Fig. 1a. Following this determination, which as previously noted consists of the injection of 0.25 g of glucose per kilo, this rat showed a daily glycosuria of 2+ for one week. The possible significance of this observation is discussed below.

The one rat, No. Y, which was given 12 daily single injections of glucose, 3.0 g per kilo, showed a persistent elevation of fasting blood sugars and marked glycosuria for 8 days after cessation of the injections—a temporary diabetic state.

It should be noted that all control animals, treated exactly as the above animals with the exception that no glucose was injected, showed no degranulation, hyperglycemia, glycosuria, or abnormal glucose tolerances.

Discussion. The injection of a large amount of glucose into the blood stream of the rat, as described above, undoubtedly causes an excessive functional demand upon the beta cells of the pancreas leading to complete exhaustion of the cells—the degranulated state. The stimulus initiating this exhaustion of the cell is of necessity the injected glucose. And from the data recorded in these experiments and from the work of Gomori and associates,[13] it is evident that the greater the concentration of the stimulating agent in the blood the greater the amount of beta cell secretion.

The beta cell in its completely exhausted state is not organically injured as determined by histologic studies—it shows no alterations other than the degranulation. However, physiologic studies as reported herein would indicate that the cell is functionally impaired. About 48 hours are required for the recovery of the cell as measured by glucose tolerance, levels of glycemia, and degree of glycosuria. Repeated injections, as exemplified by rat Y, apparently cause an accumulative functional impairment upon the beta cells; the beta cells recover within 48 hours after one injection but require one week to recover after 12 injections. Theoretically, continued injections might transform the temporary functional impairment into a permanent deficiency resulting in diabetes. This feature is being investigated.

The sensitivity of the beta cells to overstimulation probably varies somewhat from one animal to another. Rat 314 is an example of marked sensitivity to a slight stimulus following the production of functionally impaired beta cells. The day following the intra-cardiac injection of 3.0 g glucose per kilo, rat 314 had a fasting blood sugar within normal range but showed a glucose tolerance curve of a potential diabetic type, Fig. 2a. In doing the glucose tolerance 0.25 g of glucose per kilo was again injected. For one week following this procedure the rat showed a marked daily glycosuria and an elevated fasting blood sugar. Thus a small extra demand upon a previously functionally impaired

TABLE II.
Correlation of Beta Granulation with Blood Sugar, Glucose Tolerance, and Glycosuria Following One Injection of Glucose (3.0 g/kg).

Hr after injection	0	1	24	48	72	Week
Beta granulation	3	0	0-1	2-3	3	3
Avg blood glucose	118	390	163	122	108	106
Glucose tol. curve	Normal		Diabetic or Pot. diab.			Normal
Glycosuria	0	4+	2+ to 4+	0 to Tr.	0*	0*

* One exception, Rat 314, 2+ daily glycosuria for one week.

beta cell produced the picture of a temporary diabetic state.

The fact that the beta cells eventually regranulate completely is also strong evidence that the functional impairment is only temporary. The final proof, of course, for the theory of functional impairment due to overstimulation is the work of Lukens and Dohan,[9] in which cats were made permanently diabetic by the administration of large amounts of glucose.

The marked correlation between the degree of beta cell granulation with alterations of glucose tolerance, elevation of the fasting blood sugars, and the presence of glycosuria would confirm the widely held opinion that the granules are a form of insulin. The phenomenon of complete degranulation within 15 minutes in response to the stimulus of injected glucose would be further evidence for this opinion.

The addition of crystalline zinc insulin to the injected glucose resulted in an interesting observation. We had anticipated that the exogenous insulin would protect the beta cells from degranulation but interval blood sugars explained the results. The beta cells were apparently degranulated during the initial period of hyperglycemia, as shown in Fig. 1b. An initial lag in the effect of the injected insulin is well demonstrated by the blood sugar curves. The significance of this phenomenon has not yet been determined.

Summary. The beta cells of the rat's pancreas can be completely degranulated within 15 minutes by a single intra-cardiac injection of glucose. Dosage: 3.0 g glucose per kilo body weight.

The phenomenon of degranulation is reversible; regranulation follows degranulation, but requires about 48 hours.

Rats with degranulated beta cells show a reduced glucose tolerance, elevated fasting blood sugars, and glycosuria.

The addition of 3 units of crystalline zinc insulin per 1.0 g of glucose does not prevent the degranulation of the beta cells due to a delay in the effect of the injected insulin and the consequent production of a hyperglycemia.

Repeated injections of glucose cause a functional impairment of the beta cells as evidenced by the production of a temporary diabetic state.

16925

Effect of Epinephrine in Decreasing Number of Circulating Mononuclear Leucocytes in the Rat.*

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Recent evidence suggests that the number of circulating mononuclear leucocytes is under the control of adrenal cortical hormones[1,2] since these cells are decreased when an excess of cortical hormone is present and increased after adrenalectomy. Epinephrine has been reported to stimulate adrenal cortical secretion.[3-5] Epinephrine administration should, therefore, cause a decrease in the mononuclear count of the blood. Lymphopenia following epinephrine administration has recently been reported to occur in intact but not adrenalectomized dogs.[6]

The problem remains whether epinephrine changes the blood picture by direct action on the adrenal cortex or whether the mechanism is mediated through the pituitary gland. In an effort to add evidence to this point, the effect of epinephrine on the mononuclears of rat blood was determined in the presence or absence of the endocrine glands in question (pituitary and adrenal).

The results indicate that epinephrine administration is followed by a decrease in the number of circulating mononuclear leucocytes in normal, adreno-demedullated, and hypophysectomized rats but not in adrenalectomized rats.

Methods. Healthy growing male rats of the Long-Evans strain, 60 to 70 days of age and kept under similar environmental conditions were employed. They received the regular stock diet (XIV)† *ad libitum* supplemented twice weekly with fresh lettuce.

Hypophysectomy was performed under ether anesthesia. Adreno-demedullation and adrenalectomy were performed under sodium pentobarbital anesthesia. Hypophysectomized and adrenalectomized rats were used on the tenth postoperative day. Adreno-demedullated rats were used 20 or 30 days after the operation. Adrenalectomized rats were maintained on drinking water containing 1% sodium chloride.

Blood counts were made from tail vein blood. The smears were stained with Wright's stain and between 100 and 200 leucocytes were counted. White blood cells were classified as mononuclear or neutrophilic leucocytes.

Epinephrine chloride, 1:1000 (Parke Davis and Co.), was injected subcutaneously in the amount of 0.07 cc per 100 g body weight. For the purpose of control, saline solution (0.9%) was injected subcutaneously in the amount of 0.07 cc per 100 g body weight.

Results. Fifteen to 30 minutes after administering epinephrine to normal rats (Table I) a slight but consistent elevation in the number of mononuclear leucocytes occurred. After one hour, the number of mononuclears began to decrease, reaching the lowest level in 2 hours. Thereafter, the number of mononuclears began to increase, reaching the normal level after 6 to 8 hours. Neutrophilic leucocytes increased rapidly throughout the

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* Aided by grants from the Board of Research of the University of California, Berkeley, and the Rockefeller Foundation, New York City.

[1] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

[2] Reinhardt, W. O., Aron, H., and Li, C. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 19.

[3] Vogt, M., *J. Physiol.*, 1944, **103**, 317.

[4] Long, C. N. H., *Fed. Proc.*, 1947, **6**, 461.

[5] Sayers, G., and Sayers, M., *Endocrinology*, 1947, **40**, 265.

[6] Malmejac, J., Chardon, G., and Gros, A., *Bull. l'Acad. Med.*, 1946, **130**, 492.

† Diet XIV (stock diet) consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 10.0%, NaCl 0.75%, $CaCO_3$ 1.5%, hydrogenated vegetable oil (Crisco or primex) 5.25%. To each kg of diet were added 3.5 g of sardilene (fish oil concentrate containing 3000 USP units of vitamin A and 400 chick units of vitamin D per g).

TABLE I. Number of Circulating Leucocytes in the Rat After Administration of Epinephrine or Saline Solution.

	Experimental animals				Mononuclear counts Hr after injection				Neutrophil counts Hr after injection			
Treatment	Type	No.	B.W. (g)	Age (days)	0	½	2	4	0	½	2	4
Epinephrine chloride	Normal	10	233	71	11600 (1300)	11800 (1150)	5500* (750)	9000 (1600)	2900 (500)	3750 (1050)	7850* (1200)	15200* (1650)
	Adrenodemedullated	8	245	68	10400 (550)	9400 (1100)	6200* (300)	9400 (900)	2700 (350)	3300 (450)	8600* (850)	16000* (1900)
	Adrenalectomized	10	247	66	13600 (2100)	17600 (2000)	15400 (1350)	19400 (2200)	4250 (850)	4800 (550)	11300* (1250)	17100* (2200)
	Hypophysectomized	8	205	70	22000 (1400)	19200 (750)	14000* (1400)	18000 (2100)	3000 (350)	4000 (700)	5000† (650)	10900* (1250)
0.9% NaCl	Normal	10	262	69	10750 (900)	7550† (650)	7300* (600)	10650 (900)	2600 (400)	2600 (350)	3900† (400)	5250† (850)
	Adrenodemedullated	8	218	60	10150 (600)	9300 (750)	12000 (1000)	13000† (1100)	2700 (300)	3850† (300)	5050* (350)	5500* (500)
	Hypophysectomized	8	180	65	15200 (950)	15300 (800)	17700 (1000)	23000* (1400)	3200 (400)	4000 (450)	5200 (800)	9600* (950)

Leucocyte counts are expressed as the number per mm^3 (mean value with standard error in parenthesis).

* Denotes highly significant values ($p = < .01$) when compared with the 0 hour count for that group.

† Denotes probably significant values ($p = < .05$) when compared with 0 hour count for that group. Other values presented are not significant when compared in this way.

entire period. Twenty-four hours later the number of both types of leucocytes was slightly lower than the pretreatment number. Essentially the same response was obtained by injecting epinephrine into adreno-demedullated rats. Having established that the minimum level in the number of circulating mononuclear leucocytes was reached 2 hours after administering epinephrine, blood counts were restricted to a 4-hour period and were made at the intervals shown in the table.

Two hours after administering epinephrine to hypophysectomized rats, the number of mononuclear leucocytes was significantly decreased, although the percentage decrease was not as great as in the epinephrine treated normal rats. The number of neutrophils increased throughout the 4-hour period.

In adrenalectomized rats the number of mononuclear leucocytes did not fall but was slightly increased after administering epinephrine. The number of neutrophils increased as in the other groups.

In order to determine the error which might be incurred by repeated blood counts taken from the tail, and from handling the rats, 0.9% saline solution was injected into normal, adreno-demedullated, and hypophysectomized rats and blood counts were taken at the same intervals. Although the number of mononuclears was decreased in normal rats injected with saline solution, this change was not of the same magnitude as that following epinephrine injection. (It probably can be attributed to stimulation of the adrenal cortex by discharge of epinephrine resulting from excitement.) This decreased count was not observed in adreno-demedullated or hypophysectomized rats after injection of saline solution.

Statistical evaluation. The pretreatment value (0 hour) was considered as normal for the group in question and subsequent values for that group were compared with this initial count in calculating P values.[7] A change in the leucocyte count was considered highly significant if the P value was 0.01 or less and probably significant if P was 0.05 or less (Table I).

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[7] Fisher, R. A., and Yates, F., *Statistical Tables*, Oliver and Boyd, London, 1938.

It was not surprising that the initial mononuclear counts in different groups were not the same as it is well known that mononuclear leucocytes are increased after adrenalectomy or hypophysectomy. Comparison between groups, therefore, was not possible without converting all values to a percentage figure of the pretreatment count which would thus give an initial common basis for comparison. Percentage figures are not presented since this procedure did not alter the conclusions or significance of the data.

Discussion. Harvey[8] reported an increase in the mononuclear count 15 minutes after injection of epinephrine into rabbits. Bjure and Svensson[9] reported an increase after 30 minutes but a decrease after 2 hours in normal human beings. Fegler[10] reported a leucopenic phase 2 hours following administration of epinephrine to dogs. Martin[11] reported a lymphocytosis in human patients but did not make blood determinations longer than 40 minutes after injection of epinephrine. Camp[12] reported a lymphopenia but did not specify the time interval elapsing after injecting epinephrine. Harlow and Selye[13] reported a relative lymphopenia after administering epinephrine twice daily for 2 days. The effect of epinephrine in elevating the blood sugar may be related to the changes in the mononuclear counts. Following glucose administration, Elmadjian, Freeman, and Pincus[14] reported a lymphopenia which was lowest when the blood sugar was highest. This response could not be obtained in adrenalectomized rats.

Two hours after intravenous injections of epinephrine into intact dogs, Malmejac, Chardon, and Gros[6] reported a lymphopenia. The number of lymphocytes returned to the normal level after 4 to 6 hours. A slight lymphocytosis developed after injecting epinephrine into adrenalectomized dogs. These are the same time relationships which are reported in this paper following subcutaneous injection of epinephrine into rats.

The dose of epinephrine employed in the experiments reported here is admittedly too high to be within physiological limits. It is to be pointed out that epinephrine gave the responses indicated only in the presence of the adrenal gland. No attempt was made to determine the minimum effective dose to produce these effects.

Conclusions. The administration of epinephrine to normal or adreno-demedullated rats produced within 2 hours a definite fall in the number of circulating mononuclear leucocytes. The number of mononuclears did not decrease after administering epinephrine to adrenalectomized rats. This indicates that the presence of the adrenal cortex is necessary for epinephrine to cause these effects. After administering epinephrine to hypophysectomized rats, the number of mononuclears decreased as in the epinephrine treated normal rats although the percentage decrease was less. This would seem to indicate that at least one effect of epinephrine is to act directly on the adrenal cortex, releasing substances which lower the level of circulating mononuclear leucocytes.

Since this paper was submitted for publication, Gellhorn and Frank[15] have reported that epinephrine causes a reduction in the number of circulating lymphocytes in normal but not in adrenalectomized rats.

This work is the result of experiments initiated under the direction of Dr. William O. Reinhardt. Acknowledgment is made to Dr. Herbert M. Evans for advice and suggestions.

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[8] Harvey, W. H., *J. Physiol.*, 1906, **35**, 115.

[9] Bjure, A., and Svensson, J., *Studia Biologica Medica*, Sect. VI, Uppsala, 1921.

[10] Fegler, G., *Compt. Rend. Soc. Biol.*, 1927, **97**, 966.

[11] Martin, H. E., *J. Physiol.*, 1932, **75**, 113.

[12] Camp, W. J. R., *J. Lab. Clin. Med.*, 1927, **13**, 206.

[13] Harlow, C. M., and Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 141.

[14] Elmadjian, F., Freeman, H., and Pincus, G., *Endocrinology*, 1946, **39**, 293.

[15] Gellhorn, E., and Frank, S., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 426.

16926 P

Activation of Proplasmin by a Tissue Fraction.

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The proteolytic enzyme plasmin (fibrinolysin) is found in normal blood plasma in an inactive form, called proplasmin. Activation can be produced by treatment with chloroform, ether and some other organic solvents,[1] as well as by streptokinase.[2] The active form of the enzyme is occasionally found in the blood of certain types of patients,[3] but the mechanism of activation in these cases and whether there exists a physiological mechanism of activation of the enzyme in the normal organism, are at present unknown.

The evidence presented in this report indicates that an activator is present in tissue extracts which could constitute the normally occurring activator of the enzyme.

The lungs of the freshly killed adult rats were removed and forced through a 1 mm mesh. The tissue pulp was ground in a mortar, suspended in 30% sucrose solution[4] and then submitted to differential centrifugation in the cold. Tissue fractions and supernatants were obtained and labeled according to the nomenclature of Claude.[5] For testing the material, the substrate used was 0.2% fibrinogen solution in 0.01 M phosphate buffer in saline at pH 7.2, kept frozen at –10°C, prepared by the method of Ware *et al.*[6] The tissue fraction was mixed with either human blood serum or a globulin solution prepared by precipitating human blood plasma at 45% saturation of ammonium sulfate at pH 7, dissolving the precipitate in a volume of saline equal to ¼ the volume of plasma, and dialyzing against saline for 3 days. The serum or the globulin solution served as a source of proplasmin. Fibrinogen was then added and the mixture was clotted by the addition of thrombin (Lederle clotting globulin) on a glass rod. Time of dissolution of the clot at 37°C was noted.

Table I shows the activity of the different fractions from lung tissue. The presence of inhibitory action was evident in tubes 2 and 6, but no inhibitory action was noted on increasing the amount of microsome fraction (tubes 7 and 8). Control experiments showed that the tissue fractions without addition of globulin solution or the globulin solution alone had no dissolving action on the fibrin clot within the time of observation (2 hours) but occasionally the tissue fractions without addition of globulin exhibited such activity over a period of 16 to 24 hours. This may have been due to slight contamination of the fibrinogen preparation with proplasmin.

Fresh blood serum could be used instead of the globulin solution as a source of proplasmin with essentially similar results.

Conclusion and summary. The data indicate that on mixing tissue pulp from the lungs of freshly killed adult rats with blood serum or a fraction of blood serum containing proplasmin, fibrinolytic activity appeared in the mixture. This was presumably due to activation of proplasmin of serum by a tissue kinase present in the lung tissue. The kinase activity was found to be concentrated in the microsome fraction of the tissue, while the inhibitory activity was in the supernate. The

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* Senior Fellow of the Committee on Growth, National Research Council.

[1] Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 525.

[2] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

[3] Tagnon, H. J., Levenson, S. M., Davidson, C. S., and Taylor, F. H. L., *Amer. J. Med. Sc.*, 1946, **88**, 211.

[4] Hogeboom, G. H., Schneider, W. C., Palade, G. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 320.

[5] Claude, A., *J. Exp. Med.*, 1946, **84**, 61.

[6] Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Bioch.*, 1948, **13**, 231.

TABLE I.
Action of Tissue Fractions on Proplasmin.†

	Reagents in cc.*					
Tube No.	Total extr.	Mitochondria	Supernate 1	Microsomes	Supernate 2	Diss. of clot (min.)
1	.1					15
2	.3					27
3		.1				30
4		.3				35
5			.1			16
6			.3			26
7				.1		11
8				.3		9
9					.1	120+
10					.3	120+

† All fractions were suspended in a volume equal to that of the original extract.
* Fibrinogen 0.2 cc + globulin solution 0.1 cc + 0.01M phosphate buffer 0.3 cc, thrombin (on glass rod) added to each tube. Temp. 37°C; pH 7.2.
Control experiments: 1. Same as in table with omission of globulin solution: no dissolution in 2 hr. 2. Same as in table with omission of fractions of tissue: no dissolution in 2 hr.

system described here may be identical with that proposed by Astrup and Permin.[7] It is known also that the microsome fraction of lung tissue contains most of the thromboplastic activity of the total tissue extract,[5] although this should not be construed as indicating an identity between the kinase and thromboplastin.

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[7] Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.

16927

Exoerythrocytic Stages of *Plasmodium cynomolgi* in the *Macaca mulatta*.*

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The exoerythrocytic cycle from sporozoite to erythrocytic parasite has now been demonstrated in 4 species of avian malaria.[1-5] The exoerythrocytic stages, called cryptozoites and metacryptozoites by Huff and Coulston,[4] are widely distributed throughout the tissues of the avian host and develop in cells of the lymphoid-macrophage system and in endothelium.

Recently, Shortt and Garnham,[6,7] and Hawking[8] described pre-erythrocytic stages in the livers of monkeys (*Macaca mulatta*) infected with sporozoites of *P. cynomolgi*. Later Shortt, Garnham, Covell, and Shute[9] found pre-erythrocytic forms of *P. vivax* in a liver biopsy of a man on the seventh day following infection with sporozoites. In the case of *P. cynomolgi* it was suggested that the exoerythrocytic stages developed in hepatic cells, and it was reported that these parasites were not observed in any other organ or tissue of the monkey. Although Hawking, Perry, and Thurston[10] described a pre-erythrocytic parasite in the brain of a monkey inoculated intracerebrally with sporozoites, they considered this parasite to be growing in an abnormal location. Further evidence was presented by Huff and Coulston[11] indicating that the pre-erythrocytic stages of mammalian malaria may develop in cells other than hepatic cells. They inoculated sporozoites of *P. vivax* into the liver of *M. mulatta* and observed cryptozoites in cells of the liver. These cells could not be definitely identified in their preparations but appeared to be either true endothelial cells or possibly cells of the lymphoid-macrophage system.

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* This study was supported in part by a grant-in-aid from the United States Public Health Service.

[1] Huff, C. G., and Coulston, F., *Bi-monthly Report, Comm. Med. Res., Office of Scientific Research and Development*, 1943, No. 12, June 1.

[2] Reichenow, E., and Mudrow, L., *Deutsche tropenmed. Z.*, 1943, **47**, 289.

[3] Coulston, F., and Huff, C. G., *J. Infect. Dis.*, 1947, **80**, 209.

[4] Huff, C. G., and Coulston, F., *J. Infect. Dis.*, 1944, **75**, 231.

[5] Huff, C. G., Coulston, F., Laird, R. L., and Porter, F. J., *J. Infect. Dis.*, 1947, **81**, 7.

[6] Shortt, H. E., and Garnham, P. C. C., *Nature*, London, 1948, **161**, 126.

[7] Shortt, H. E., and Garnham, P. C. C., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1948, **41**, 785.

[8] Hawking, F., *Nature, London*, 1948, **161**, 175.

Materials and methods. Studies on the exoerythrocytic cycle of *P. cynomolgi* in *M. mulatta* have recently been initiated in this laboratory. The strain of *P. cynomolgi* used was received from Dr. R. J. Porter, School of Public Health, University of Michigan, in June 1946. Sporozoites were obtained from *Anopheles quadrimaculatus* which had been fed 15-19 days previously upon infected monkeys.

In these experiments a total of 12 monkeys have been used; 9 were infected with sporozoites, and 3 were controls. Pertinent data on these animals are presented in Table I. In some cases mosquito salivary glands, heavily infected with sporozoites, were inoculated directly into the liver.[4] Afterwards, this marked area was biopsied and serially sectioned. Sporozoites for intravenous inoculations were obtained by grinding in a glass mill either the thoraces of mosquitoes with intact salivary glands or the dissected salivary glands. A series of control experiments was done in which normal mosquito tissue was introduced into monkeys in the same manner as the infected material mentioned above.

The monkey tissues were fixed in Zenker-formalin, embedded in paraffin, and stained by either the Maximow method[4] or the modified Giemsa method described by Shortt and Cooper.[12]

Results. As a result of these investigations, pre-erythrocytic stages of *P. cynomolgi* have been found in the liver and spleen of *M. mulatta*. The experimental data are presented in Table I.

In *Exp. 1*, a monkey was inoculated intravenously with the ground thoraces of 178 heavily infected mosquitoes and was sacrificed and necropsied 6 days and 20 hours later. Upon histological examination of the tissues, several small segmenters and schizonts were observed in the liver in Kupffer cells (Fig. 1) and in the spleen in large mononuclear phagocytic cells, probably reticular in nature. No parasites were found in cells which could definitely be called hepatic cells, but this should not rule out the possibility that such cells may have been infected.

In *Exp. 2*, a monkey was inoculated twice directly into the liver with 18 and 24 heavily infected salivary glands. Biopsies of these inoculation sites, at 47 hours and at 89 hours, disclosed a few schizonts (12-18 μ) some of which appeared to be in Kupffer cells. These pre-erythrocytic stages were always found near the site of inoculation.

Nine days and 18 hours after inoculation, the day following detectable parasitemia, another liver biopsy was taken above the 47-hour inoculation site. In this specimen numerous parasites were found in the liver sinusoids in a circumscribed area. These stages were either schizonts and segmenters or merozoites free in the lumen of the sinusoid and were often associated with the Kupffer and littoral cells. Other parasites

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[9] Shortt, H. E., Garnham, P. C. C., Covell, G., and Shute, P. G., *Brit. Med. J.*, 1948, **1**, 547.

[10] Hawking, F., Perry, W. L. M., and Thurston, J. P., *Lancet*, 1948, **ccliv**, 783.

[11] Huff, C. G., and Coulston, F., *J. Parasitol.*, 1948, **34**, 264.

[12] Shortt, H. E., and Cooper, W., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1948, **41**, 427.

TABLE I.

Biopsies and Necropsies of Normal Monkeys and Monkeys Infected with Sporozoites of *P. cynomolgi.*

Exper. No.*	Mode of sporozoite inoculation	No. of infected or uninfected mosquitoes used	Site of biopsy	Time of tissue collection				Pre-erythrocytic stages present	Remarks‡
				Between inoculation and biopsy		Between inoculation and necropsy†			
				days	hr	days	hr		
				Infected Monkeys.					
1 A	Intravenous	178				6	20	Yes	Schizonts and few segmenters. Some in spleen.
2 A	Liver	24	Liver	1	23			Yes	Few schizonts. Parasitemia on 8th day.
B	,,	18	,,	3	17			Yes	Few schizonts.
C			,,	9	18			Yes	Segmenters; biopsy taken near 2 A.
D			,,	9	18			No	Biopsy of normal lobe.
E			Spleen	11	18			?	Large bodies resembling segmenters.
3 A	,,	28	Liver	3	22			Yes	Schizonts and a few segmenters. Parasitemia on 9th day.
B	,,	27	,,	6				No	Injection site not located.
C						14		—	Unstudied.
4 A	Intrav.	223	,,	1	20			Yes	Small trophozoites.
B						5	19	Yes	Schizonts in liver smears.
				Uninfected Monkeys.					
5 A		0	,,					No	Biopsy of normal monkey.
B			Spleen					No	
6 A	Liver	20	Liver	2	3			No	Control to Exp. 2 and 3.
B	,,	29	,,	4	1			No	Control to Exp. 2 and 3.
C			,,	10	1			No	Biopsy near 6 A.
D			Spleen	11	2			No	Tip of spleen.
7 A	Intrav.	180				5	20	No	Control to Exp. 1 and 4.

* Each experiment represents one monkey.

† All monkeys were sacrificed and tissue collected.

‡ All parasites are in liver unless otherwise noted.

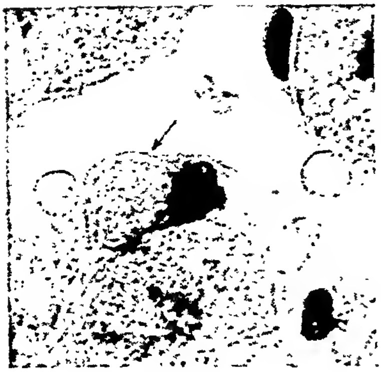

FIG. 1.

A pre-erythrocytic parasite of *P. cynomolgi* in a Kupffer cell, 6 days and 20 hours following the intravenous inoculation of sporozoites. Liver fixed in Zenker-formalin and stained by Maximow's hematoxylin, eosin-azur method. ×1300.

were found in cells which could not be identified; these may have been hepatic cells.

A splenic biopsy was also performed on this animal, 11 days and 18 hours after inoculation. Large bodies resembling schizonts and segmenters were observed in the venous sinuses, the pulp veins, and the trabecular veins. Despite this resemblance, the similarity of these forms to white thrombi raises a serious question as to whether they were exoerythrocytic stages. Heterophils, eosinophils, small and large lymphocytes, and monocytes were often observed surrounding and lying within these forms, resembling the leucocytic reaction which earlier investigators[7,10] had associated with segmenting exoerythrocytic stages of *P. cynomolgi*.

In *Exp. 3*, the liver of a monkey was inoculated in 2 places—in one site with 28 and in the other site with 27 heavily infected salivary glands. Liver biopsies were performed at 94 hours and at 144 hours but the inoculation site was located microscopically only in the 94-hour specimen. Schizonts and an occasional pre-segmenter were found in the 94-hour biopsy and ranged in length from 15-38 μ. These pre-erythrocytic parasites were primarily in Kupffer cells; some were definitely in hepatic cells. Three parasites were observed in the inoculation area in cells that could only be classified as large mononuclear phagocytic cells, similar to macrophages and Kupffer cells. The reaction area at the inoculation site was composed chiefly of degenerating liver cells, mosquito debris, heterophils, eosinophils, and a large variety of mononuclear phagocytic cells typical of an active inflammatory host response.

In *Exp. 4*, a monkey was inoculated intravenously with the ground thoraces of 223 heavily infected mosquitoes and was sacrificed 5 days and 19 hours later. Liver and spleen biopsies were made at 44 and 87 hours after inoculation. Air dried, Giemsa stained smears of the liver made at necropsy revealed the presence of 5 exoerythrocytic parasites, the largest of which had 26 chromatin bodies, the smallest 5. As is usual with such preparations, it was not possible to determine the type of host cell in which these parasites were located.

The parasites in these air dried liver smears contained vacuoles similar to those described for *P. cynomolgi*[7] and *P. relictum matutinum*.[13] However, no large vacuoles were seen in the histological preparations fixed in Zenker-formalin in any of the above experiments: clefts were observed.

In the biopsy made at 44 hours, small parasites resembling the small metacryptozoites of avian malaria were found in the Kupffer cells. This observation together with the findings of Experiment 2 suggest that cryptozoic segmentation occurred at about the 40th to the 48th hour.

A series of *control experiments* was performed to determine whether the observations mentioned above could be at all related to the parenteral inoculation of normal mosquito tissue. In this work, monkeys were inoculated, biopsied, and necropsied like the animals which received infected material. Tissues obtained from these animals were treated in the same manner as those from the infected monkeys. *Experiment 5* served as a normal control for the biopsies of infected liver and spleen. In *Exp. 6*, normal salivary glands were inoculated directly into the liver and then biopsied; this controlled Experiments 2 and 3. The monkey in *Exp. 7* controlled the infections, produced intravenously, in the animals of Exp. 1 and 4.

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[13] Manwell, R. D., *Am. J. Trop. Med.*, 1940, 20, 859.

In none of these controls were bodies found which were similar to the pre-erythrocytic stages observed in the infected animals. The control material did demonstrate the need for caution in interpreting the data, for in these animals there were some forms such as degenerating liver cells and thrombi which might have been mistaken for parasites.

Discussion. The observations reported above suggest that the morphological characteristics of the pre-erythrocytic stages of *P. cynomolgi* are remarkably like those of the pre-erythrocytic stages of avian malaria. Certainly the tissue stages of *P. cynomolgi* are more similar to those of *P. gallinaceum* than of *Hepatocystes* (*Plasmodium*) *kochi*.[14] The exoerythrocytic stages of *Hepatocystes* resemble those stages observed in *Leucocytozoan* infections of ducks[15] while the *P. gallinaceum* and *P. cynomolgi* stages appear much like those of *Hemoproteus*. An obvious difference exists in that hepatic cells are not invaded by avian malaria and *Hemoproteus* whereas they may be in simian malaria, *Hepatocystes* and *Leucocytozoan*. Since *P. mexicanum* in the lizard[16] has both elongatum and gallinaceum type exoerythrocytic stages, it is to be expected that other species of malaria could have diverse host cell developmental potentialities.

Summary. Pre-erythrocytic stages of *P. cynomolgi* have been observed in Kupffer cells, hepatic cells, and mononuclear phagocytic cells of the liver, as well as in large mononuclear phagocytic cells of the spleen, probably reticular in nature.

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[14] Garnham, P. C. C., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1948, **41**, 601.

[15] Huff, C. G., *J. Infect. Dis.*, 1942, **71**, 18.

[16] Thompson, P. E., and Huff, C. G., *J. Infect. Dis.*, 1944, **74**, 48.

16928

Determination of Carbon 14 in Fatty Acids by Direct Mount Technic.*

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The measurement of C^{14} in fatty acid fractions prepared from animals into which a C^{14}-labeled fatty acid has been introduced is beset with considerable difficulty. A sample of high specific activity is not often obtained because the administered radioactive fats are diluted by a factor of at least 1000 when mixed with the body pool of fatty acids. The activity per unit of mass is further reduced by a factor of 17 when the conventional $BaCO_3$ technics are used. The first dilution—that due to mixing with the body pool of fatty acids—obviously cannot be avoided. In order, however, to circumvent a further reduction of the activity, it seemed desirable to investigate direct mounting of fat samples.

When samples of C^{14}-containing fatty acids were mounted directly on bare aluminum discs, reproducible values for counting rates were not obtained because the material collected on the surface of the discs in the form of globules. Reproducible results were obtained by employing a cover of lens paper which brought about an even distribution of the fatty acids on the disc. The degree of reproducibility that can be attained by mounting radioactive fatty acids on lens paper-covered aluminum discs is shown in Table I which records the results of quadruplicate determinations for each of 3 different fatty acid samples. The deviations from the averages did not exceed 5%. The degree of reproducibility which can be at-

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* Aided by a grant from the American Cancer Society (recommended by the Committee on Growth).

TABLE I.

Reproducibility of C^{14} Counting When Fat Was Mounted on Lens Paper-covered Aluminum Discs.

(In each experiment 4 one-cc aliquots of a solution of C^{14}-labeled palmitic acid in corn oil were separately mounted.)

Fatty acid sample	Counts per minute					% deviation from avg activity
	Per mount				Avg	
1	2780	2790	2780	2750	2775	0.5
2	3720	3610	3610	3810	3690	2.1
3	4430	4500	4380	4460	4440	0.8

tained in the counting of C^{14} by this method, when employed by different individuals was also investigated and it was found that the variation in the counts obtained by 3 individuals did not exceed 5%.

Although the method described above was designed primarily to avoid the dilution of activity inherent in the preparation of $BaCO_3$ mounts, its simplicity also permits a considerable saving of time. Fifty or more samples can be readily mounted by a single individual in a period of 8 hours.

A comparison of C^{14} activity as measured by direct mount technic with that measured as $BaCO_3$. It is frequently necessary to compare the specific activity of a fatty acid fraction with that of a non-lipid fraction or that of expired CO_2. Since at present the activity in the two latter materials is usually determined after conversion to $BaCO_3$, it became necessary to relate the activity of fat as measured by the direct mounting technic described below to its activity when measured in the form of $BaCO_3$.

Corn oil fatty acids were mixed with a sample of C^{14}-labeled palmitic acid and the mixture dissolved in petroleum ether. Suitable dilutions were then prepared so that each one-cc aliquot contained from 2.5 to 35 mg of fatty acids. Each sample was directly mounted and its activity determined. Duplicate samples were oxidized to CO_2 and converted to $BaCO_3$ and the activity of the $BaCO_3$ determined. All $BaCO_3$ counts were corrected to a standard mass of 40 mg according to the method of Henriques *et al.*[1]

The values for the ratio:

$$\frac{\text{Corrected counts from BaCO}_3}{\text{Counts from directly mounted fatty acids}}$$

were determined for many samples. A plot of these values as ordinates against mg of fatty acids used in the direct mount as abscissa is shown in Fig. 1. From this empirical curve, the factor for converting counts obtained from a direct mount to the activity that would have been obtained had the C^{14} of the fatty acids been measured after conversion to $BaCO_3$ can be read.

In order to test the reliability of the conversion factors, 5 samples of tissue fatty acids isolated from rats that had received C^{14}-labeled palmitic acid were mounted directly in duplicate and the activities determined. The values obtained were then converted to the $BaCO_3$ basis by using the factors in Fig. 1. Aliquots of these same 5 samples were then oxidized to CO_2 and their activities determined in the form of $BaCO_3$. The results are shown in Table II.

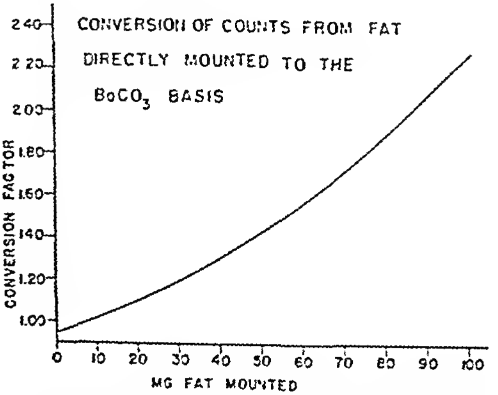

FIG. 1.

Factors for conversion of activity as measured by direct mounting technic to activity on a $BaCO_3$ basis.

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[1] Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., *Ind. Eng. Chem. Anal. Ed.*, 1946, **18**, 349.

animals of Exp. 1 and 4.

In none of these controls were bodies found which were similar to the pre-erythrocytic stages observed in the infected animals. The control material did demonstrate the need for caution in interpreting the data, for in these animals there were some forms such as degenerating liver cells and thrombi which might have been mistaken for parasites.

Discussion. The observations reported above suggest that the morphological characteristics of the pre-erythrocytic stages of *P. cynomolgi* are remarkably like those of the pre-erythrocytic stages of avian malaria. Certainly the tissue stages of *P. cynomolgi* are more similar to those of *P. gallinaceum* than of *Hepatocystes (Plasmodium) kochi.*[14] The exoerythrocytic stages of *Hepatocystes* resemble those stages observed in *Leucocytozoan* infections of ducks[15] while the *P. gallinaceum* and *P. cynomolgi* stages appear much like those of *Hemoproteus.* An obvious difference exists in that hepatic cells are not invaded by avian malaria and *Hemoproteus* whereas they may be in simian malaria, *Hepatocystes* and *Leucocytozoan.* Since *P. mexicanum* in the lizard[16] has both elongatum and gallinaceum type exoerythrocytic stages, it is to be expected that other species of malaria could have diverse host cell developmental potentialities.

Summary. Pre-erythrocytic stages of *P. cynomolgi* have been observed in Kupffer cells, hepatic cells, and mononuclear phagocytic cells of the liver, as well as in large mononuclear phagocytic cells of the spleen, probably reticular in nature.

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[14] Garnham, P. C. C., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1948, **41**, 601.

[15] Huff, C. G., *J. Infect. Dis.*, 1942, **71**, 18.

[16] Thompson, P. E., and Huff, C. G., *J. Infect. Dis.*, 1944, **74**, 48.

16928

Determination of Carbon 14 in Fatty Acids by Direct Mount Technic.*

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The measurement of C^{14} in fatty acid fractions prepared from animals into which a C^{14}-labeled fatty acid has been introduced is beset with considerable difficulty. A sample of high specific activity is not often obtained because the administered radioactive fats are diluted by a factor of at least 1000 when mixed with the body pool of fatty acids. The activity per unit of mass is further reduced by a factor of 17 when the conventional $BaCO_3$ technics are used. The first dilution—that due to mixing with the body pool of fatty acids—obviously cannot be avoided. In order, however, to circumvent a further reduction of the activity, it seemed desirable to investigate direct mounting of fat samples.

When samples of C^{14}-containing fatty acids were mounted directly on bare aluminum discs, reproducible values for counting rates were not obtained because the material collected on the surface of the discs in the form of globules. Reproducible results were obtained by employing a cover of lens paper which brought about an even distribution of the fatty acids on the disc. The degree of reproducibility that can be attained by mounting radioactive fatty acids on lens paper-covered aluminum discs is shown in Table I which records the results of quadruplicate determinations for each of 3 different fatty acid samples. The deviations from the averages did not exceed 5%. The degree of reproducibility which can be at-

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* Aided by a grant from the American Cancer Society (recommended by the Committee on Growth).

The flame was removed and CO_2-free air passed through the apparatus for an additional 10 minutes. The $Na_2C^{14}O_3$-NaOH was next forced into a volumetric flask by means of air pressure. Aliquots were taken for the precipitation and mounting of $BaCO_3$ and for the determination of CO_2.

The completeness of oxidation under the conditions described above was tested for the following compounds: palmitic acid, cholesterol, glucose, and corn oil. Six mg of the first 3 compounds were used whereas corn oil was tested over a range of 5 to 30 mg. In all cases, the recovery of CO_2 was from 95 to 100% of the theoretical.

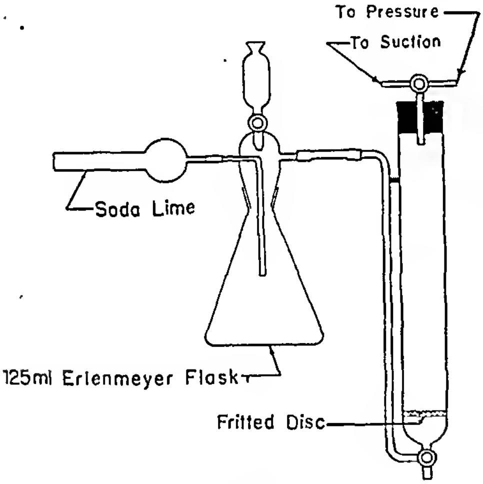

FIG. 2.
Apparatus used for oxidation of organic compounds.

Determination of CO_2. An excess of a saturated $BaCl_2$ solution was added to aliquots of the $Na_2C^{14}O_3$ solution. This solution was then titrated with dilute HCl to the phenolphthalein end point. Brom cresol green was then added, and the solution titrated with standard 0.1 N HCl to the new end point. The amount of $BaCO_3$ precipitated was calculated from the titration difference which had been corrected for a blank titration value.

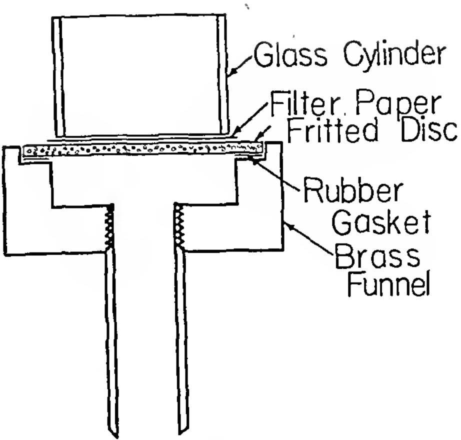

FIG. 3.
Filtration apparatus for preparation of $BaCO_3$ mounts. The filter paper used is Whatman No. 42. The glass cylinder is held firmly in place by means of two elastic bands attached to glass ears on the sides of the cylinder and to metal screws on the sides of the brass funnel.

Preparation of the $BaCO_3$ mount.† An excess of a saturated $BaCl_2$ solution was added to an aliquot of the $Na_2C^{14}O_3$ solution. Suction was applied to the filtration apparatus (Fig. 3) and the suspension of $BaCO_3$ poured into the glass cylinder. The suspension was allowed to filter completely and the precipitate, while still moist, was washed first with water and next with acetone. Suction was maintained constantly. After removal of the glass cylinder, an infra-red lamp was placed one inch above the surface of the mount and hot air was pulled through the mount for several minutes. Suction was discontinued at this point. The paper and $BaCO_3$ were removed as a flat mount. (There should be little or no curvature.) For determination of its radioactivity, the mount was held flat by means of the carrier shown in Fig. 4. No loss of activity has been observed in samples kept in covered lucite trays for as long as 11 weeks.

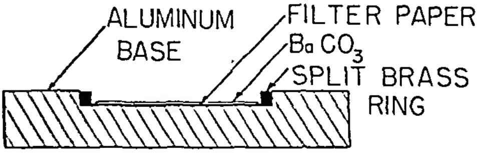

FIG. 4.
Holder for keeping $BaCO_3$ filter paper mounts flat during counting.

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† Similar mounting technics have been described for benzidine sulfate[1] and P32 in plant material.[4]

[4] MacKenzie, A. J., and Dean, L. A., *Anal. Chem.*, 1948, **20**, 559.

TABLE II.
Reliability of Empirical Conversion from Counts per Minute per Mg Tissue Fatty Acids to Counts per Minute per Mg $BaCO_3$.

1	2	3
	Counts per Minute per Mg $BaCO_3$.	
Mg fatty acids	*Calc. from direct mount by use of factors in Fig. 1*	*Found by oxidation of fatty acids to $BaCO_3$.*
5.7	10.8	10.3
5.7	10.6	10.2
12.9	10.8	10.3
13.3	10.4	10.1
17.9	11.0	11.1
18.1	10.6	10.3
22.5	10.9	10.3
22.9	11.0	10.3
28.1	10.9	10.5
27.9	11.1	10.1

The values obtained by this conversion (col. 2) are in good agreement with those obtained by direct oxidation of the fatty acids to $BaCO_3$ (col. 3). This agreement, therefore, justifies the use of the conversion factors and the determination of the radioactivity in fatty acids by the direct mount technic, a procedure that yields the highest activity per unit of mass.

Experimental. Direct mounting of fatty acids on lens paper-covered aluminum discs. An aluminum disc 1.75 inches in diameter, lined with a piece of lens paper of the same diameter, was weighed and then placed approximately 6 inches below an infra-red lamp. An aliquot of a fatty acid solution (usually one cc) containing the C^{14}-labeled fatty acid was added dropwise to the warmed lens paper at a rate that kept the surface constantly and uniformly wet but prevented the fat solution from creeping beyond the edge of the disc. The disc and its contents were then reweighed. It was found that, by this method, about 40 mg could be safely mounted on a single disc without encountering loss from creeping. If a concentrated solution of fatty acids in ether is evaporated by using an air stream instead of an infra-red lamp, however, as much as 150 mg of fatty acids can be mounted on a disc. The activity of the mounted material was measured by a thin mica window Geiger tube.

Satisfactory results with lens paper which spreads the solution evenly have been reported by Fager.[2] His procedure, however, differs from that described above.

Wet combustion of fatty acids. The apparatus (Fig. 2) used for wet oxidation is a modification of that described by Skipper *et al.*[3] Potassium iodate was omitted from the combustion fluid. Concentrated H_2SO_4 or the combustion fluid was used as a joint lubricant. The tapered joints of the standard glass-stoppered 125 cc Erlenmeyer flasks occupy a portion of the 19/38 joints (Fig. 2). The samples were pipetted into the flasks and the solvent evaporated on the steam bath, the last traces being blown out with nitrogen. After the apparatus was assembled, the vacuum line was opened and CO_2-free air drawn through at a rate sufficient to provide the necessary dispersion in the NaOH tower. A measured amount of the combustion fluid was then added through the funnel, and the flask heated gently until fumes first appeared. This temperature, or a slightly lower one, was maintained for 5 minutes. Care was taken to avoid the excessive production of SO_3 fumes.

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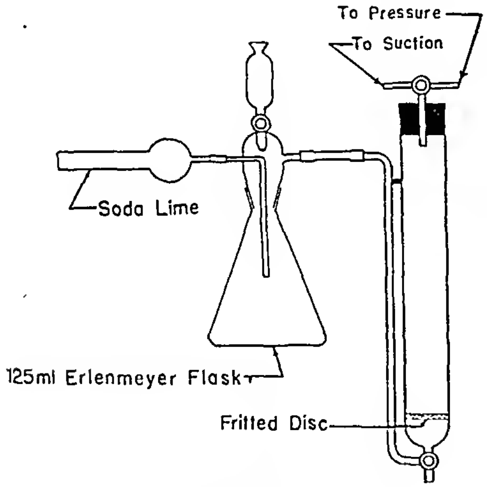
[2] Fager, E. W., Reported in Symposium on the Use of Isotopes in Biological Research, University of Chicago, March, 1947.

[3] Skipper, H. E., Bryan, C. E., White, L., Jr., and Hutchinson, O. S., *J. Biol. Chem.*, 1948, 173, 371.

The flame was removed and CO_2-free air passed through the apparatus for an additional 10 minutes. The $Na_2C^{14}O_3$-NaOH was next forced into a volumetric flask by means of air pressure. Aliquots were taken for the precipitation and mounting of $BaCO_3$ and for the determination of CO_2.

The completeness of oxidation under the conditions described above was tested for the following compounds: palmitic acid, cholesterol, glucose, and corn oil. Six mg of the first 3 compounds were used whereas corn oil was tested over a range of 5 to 30 mg. In all cases, the recovery of CO_2 was from 95 to 100% of the theoretical.



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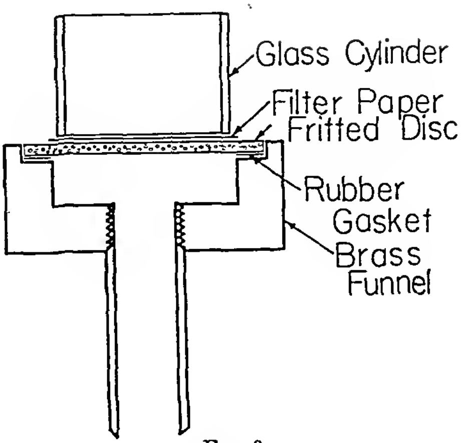

Fig. 3.
Filtration apparatus for preparation of $BaCO_3$ mounts. The filter paper used is Whatman No. 42. The glass cylinder is held firmly in place by means of two elastic bands attached to glass ears on the sides of the cylinder and to metal screws on the sides of the brass funnel.

Preparation of the $BaCO_3$ mount.† An excess of a saturated $BaCl_2$ solution was added to an aliquot of the $Na_2C^{14}O_3$ solution. Suction was applied to the filtration apparatus (Fig. 3) and the suspension of $BaCO_3$ poured into the glass cylinder. The suspension was allowed to filter completely and the precipitate, while still moist, was washed first with water and next with acetone. Suction was maintained constantly. After removal of the glass cylinder, an infra-red lamp was placed one inch above the surface of the mount and hot air was pulled through the mount for several minutes. Suction was discontinued at this point. The paper and $BaCO_3$ were removed as a flat mount. (There should be little or no curvature.) For determination of its radioactivity, the mount was held flat by means of the carrier shown in Fig. 4. No loss of activity has been observed in samples kept in covered lucite trays for as long as 11 weeks.

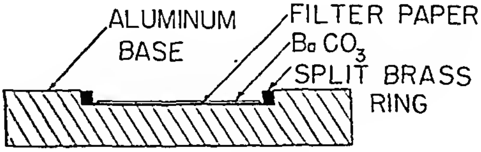

Fig. 4.
Holder for keeping $BaCO_3$ filter paper mounts flat during counting.

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† Similar mounting technics have been described for benzidine sulfate[1] and P^{32} in plant material.[4]

[4] MacKenzie, A. J., and Dean, L. A., *Anal. Chem.*, 1948, **20**, 559.

TABLE III.
Reproducibility of C^{14} Determination When $BaC^{14}O_3$ Was Mounted on Filter Paper.

Sample	$BaCO_3$ mounted, mg	Counts per min. Observed	Counts per min. Corrected for mass	Specific activity of $BaCO_3$, counts per min. per mg	% difference between duplicate specific activities*
A	57.3	725	860	15.0	4.5
A	57.8	765	910	15.7	
B	76.1	1370	1920	25.2	1.6
B	74.5	1330	1850	24.8	
C	54.5	925	1060	19.5	4.5
C	53.4	955	1090	20.4	
D	43.5	329	342	7.9	3.9
D	44.1	323	336	7.6	
E	43.4	143	149	3.4	2.9
E	43.0	148	152	3.5	

* These values include errors of titration and counting.

After its radioactivity had been determined, the $BaCO_3$ mount was transferred to an Erlenmeyer flask and an excess of standard 0.1 N HCl added. After the reaction was complete, the excess acid was titrated to the brom cresol green end point with standard alkali. The weight of the $BaCO_3$ was calculated from the equivalents of acid consumed.

For permanent mounts, or as an alternative to the $BaCO_3$ titration procedure, the filter paper can be weighed before and after the $BaCO_3$ is mounted. For samples of small weight, the filter paper should be dried at 120° before each weighing.

In order to test the reliability of the mounting of $BaCO_3$ on filter paper, the following experiments were carried out. Five $Na_2C^{14}O_3$ solutions (A to E, Table III) of varying radioactivity were used. From each solution, 2 mounts were prepared on filter paper (as described above) and their C^{14} activity determined. The weight of the $BaCO_3$ of each mount was calculated from titration values. The 2 values found for the specific activity of each sample (Table III) were in good agreement, no pair differing by more than 5%.

The data presented here apply only to the particular geometry of the counting device employed in this laboratory and hence cannot be applied to cases where the counting arrangements differ.

Summary. It is demonstrated in the present investigation that the C^{14} activity of a fatty acid sample can be readily determined by a direct procedure that avoids the dilution of activity and laboriousness associated with the preparation of $BaCO_3$ mounts. The simplicity of this new procedure permits a single operator to mount as many as 50 samples in 8 hours with an error of reproducibility not in excess of 5%. By means of an empirically constructed curve, the observed activities can be converted to a $BaCO_3$ basis.

16929

Leucocyte Blockade of *in vitro* Tuberculin Cytolysis.*

Cutting B. Favour. (Introduced by J. H. Mueller.)

From the Medical Clinic, Peter Bent Brigham Hospital, and the Department of Medicine, Harvard Medical School.

It has recently been shown that a portion of the white blood cells taken from a person acutely ill with tuberculosis are "lysed" by amounts of tuberculin not toxic for white blood cells taken from normal subjects or from individuals suffering from a variety of other acute illnesses.[1] It has also been found that this cytotoxic effect of tuberculin involves only the lymphocyte in the mouse,[2] a species which shows no intracutaneous sensitivity to tuberculin[3] whereas both lymphocytes and granulocytes are destroyed *in vitro* in the blood of man and guinea pigs showing *in vitro* cytolysis. A possible explanation for this discrepancy may be a difference in the affinity of human and mouse white blood cells for tuberculin. The experiments to be described are presented to show that indeed human white blood cells do and mouse white blood cells do not adsorb tuberculin.

Materials. 1. Mouse tuberculosis. Six-week-old albino Swiss mice were inoculated intravenously with 0.1 cc of a 6 to 10 day culture of H37Rv grown in synthetic media.[4] In this experiment 60% of the animals died within 6 weeks. Those surviving at 2 to 4 months were individually exsanguinated from the heart and the spleens from selected mice with gross evidence of advanced pulmonary tuberculosis removed and minced in an 0.85% saline solution containing 200 mg% glucose. Cell suspensions free of tissue fragments were washed three time by centrifugation and resuspension in the same solution and the cell count adjusted to 200,000 cells per cu. mm.

2. *Human tuberculosis patients* hospitalized for acute tuberculous infections were bled without stasis from the antecubital vein into a syringe containing 1 ml (10 mg) of heparin solution (Roche Organon). The 20 ml of blood was allowed to stand at room temperature in a test tube placed at an angle of 20-30°. Within 20 minutes the sedimentation was sufficient to give an almost quantitative separation of red cells from the plasma. The plasma, containing the majority of the white blood cells, was removed and centrifuged lightly to sediment the leucocytes. The supernatant containing most of the platelets was removed, centrifuged for 20 minutes at 2000 RPM to remove the platelets and its supernatant used to resuspend the homologous white blood cells. The cell concentration was adjusted to 200,000 cells per cu. mm.

3. *Normal mouse* white cells were obtained in the same fashion as tuberculous mouse white cells. Normal human white cells were obtained in a similar manner to that used for cells from tuberculous patients. A rapid sedimentation rate was induced in the normal human blood after heparinizing by adding 2 ml of a physiological solution of fibrinogen solution† per 20 ml of blood.

4. *Tuberculin.* O. T. obtained from the Massachusetts Department of Health was dialyzed against three changes of 0.85% saline during 3 days and the volume adjusted so that the concentration used represented a 6-fold dilution of the original material.

Heparinized blood from normal and tuberculous mice and humans was obtained by cardiac and venipuncture respectively immediately prior to setting up the experiment.

Method. A sample protocol illustrates the

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* Work done under a U.S.P.H.S. Research Grant.

[1] Fremont-Smith, P., and Favour, C. B., Proc. Soc. Exp. Biol. and Med., 1948, 67, 502.

[2] Favour, C. B., Proc. Soc. Exp. Biol. and Med., 1947, 65, 269.

[3] Gerstl, B., and Thomas, R. M., *Yale J. Biol. and Med.*, 1940-41, 13, 679, ref. cit.

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, 83, 409.

† Fifteen mg of the lyophilized Fraction I of Cohn per ml of distilled water.

	Normal			Tuberculous		
	T_1	S_1	P_1	T_2	S_2	P_2*
Normal cells	.8 ml	.8				
” plasma			.5			
Tuberculous cells				.8	.5	
” plasma						.5
O.T.	.2		.2	.2		.2
Saline		.2			.2	

Incubated at 37°C for 30 min.
T_1, S_1, T_2, S_2 centrifuged 10 min. at 2000 RPM.
The supernatants removed and used in procedure below.

	I	II	III	IV	V	VI	VII	VIII	IX
Man:									Normal blood
Heparinized tuberculous blood	.4 ml	.4	.4	.4	.4	.4	.4	.4	.4
Supernatant T_1	.1								
” S_1		.1							
” P_1			.1						
” T_2				.1					
” S_2					.1				
” P_2						.1			
O.T. 1:2							.1		.1
Saline								.1	
WBC 5′	4530	4020	4440	4670	4520	4290	4000	4020	3140.
WBC 60′	4400	4000	2880	4550	4340	3430	2610	3940	3010
% difference	−3.0	0	−35.1	−2.5	−3.8	−20.7	−34.7	−2.1	−2.1
Mouse:									Normal blood
Spleen cells	5020	4280		5100	4390		5100	4770	2700
	3390	4340		3450	4420		3360	4770	2700
	−33.5	+1.2		−32.3	−0.8		−34.0	0	0

* T = tuberculin + cells.
S = saline + cells.
P = tuberculin + plasma.
1 = normal.
2 = tuberculous.

method of experimentation. The tubes and counting procedures used were the same as described in a recent communication.[5] In the experiments using human blood, subjects with the same blood groups were used.

The illustrated experiment has been carried out 2 or more times on 4 different subjects and 3 separate pools of mouse spleen cells with essentially identical findings. In many other experiments done to explore the optimal concentrations of various ingredients, it has been found that the cell concentrations, amounts of O.T. used, and the duration of incubation are critical. Although complex, the experiment can be repeated regularly when the indicated precautions are observed.

"Full strength" O.T. is used in the first section to equal in final dilution that obtained with O.T. 1:2 in the second section.

Results. Inspection of the table indicates that the plasma suspension of white blood cells of normal and of tuberculous people blocks tuberculin when an *in vitro* biological assay method for the detection of tuberculin is used. White cells from the spleen of a tuberculous mouse do not so block tuberculin. In neither species does the presence or absence of tuberculosis in the donor host alter the type of *in vitro* cell-tuberculin-blockade. It is interesting, however, that the serum of a normal person does not inactivate tuberculin whereas the tuberculous individual's blood does partially block the subsequent *in vitro* cytotoxic effect of tuberculin.

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[5] Favour, C. B., and Fremont-Smith, P., to be published.

Comment. It has long been assumed that the tuberculin which is placed in the skin or other tissues and which produces a delayed tuberculin reaction is localized in that area. The very nature of the tuberculin reaction itself is evidence for this belief. In the experiments reported here some direct evidence for this belief is presented. The leucocytes of man which are the classic model of host cells of the tissue culture worker are capable of reacting with tuberculin in such a way that it no longer has cytotoxic effects on sensitized white cells from tuberculous subjects. Mouse leucocytes, on the other hand, do not react with tuberculin. It is perhaps not a coincidence that the tuberculous mouse shows no intracutaneous reactions to tuberculin[3] as do tuberculous rabbits, guinea pigs and humans. These findings suggest that there is a primary "affinity" for tuberculin of the tissues of some species and not for others. This "affinity" does not depend on the presence of a tuberculous infection but is present in those species capable of developing a tuberculin reaction. This phenomenon may be compared to other tissue tropisms, for example the neurotropic viruses. It also helps to explain some of the differences. in cell damage produced in the tuberculin type reaction as contrasted to the cellular changes in anaphylactic phenomenon.

Summary. 1. Suspensions of human white blood cells in homologous fresh plasma will adsorb tuberculin (O.T.) from the plasma.

2. White blood cells from normal persons with a negative intracutaneous reaction to tuberculin as well as white cells from persons with active tuberculosis show this phenomenon.

3. Suspensions of mouse white blood cells in homologous fresh plasma will not adsorb tuberculin from the plasma.

4. There is no difference in the lack of tuberculin adsorption by white blood cells from normal or tuberculous mice.

5. Normal human plasma does not whereas plasma from patients with active tuberculosis does partially inactivate tuberculin under the conditions of these experiments.

6. Tuberculin has been assayed by its *in vitro* cytotoxic effect on white blood cells from tuberculous subjects.

16930

Failure of Phenosulfazole to Influence the Course of Infection with Murine Poliomyelitis Virus in Mice.

CLAUS W. JUNGEBLUT.

From the Department of Bacteriology, Columbia University College of Physicians and Surgeons, New York City.

Sanders, SubbaRow, and Alexander[1] presented evidence to show that a new sulfonamide compound (N-(2-thiazolyl)-phenol sulfonamide, abbreviated as phenosulfazole, trade name "Darvisul") which was synthesized at the Calco Chemical Division of the American Cyanamid Company acted as an effective antiviral substance in mice infected with the Col. SK strain of murine poliomyelitis virus. Drug treatment instituted 24 hours following intraperitoneal injection with virus at various levels of potency apparently brought about a regular and striking reduction in the mortality rate when compared with that among untreated controls. As judged from the protocols, however, the therapeutic effect showed no clear gradations with respect to amount of virus used for infection or

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[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Rep. on Biol. and Med.*, 1948, 6, 385.

TABLE I.
Attempted Chemotherapy of Murine Poliomyelitic Infection with Phenosulfazole.

Exper.	Seed virus	Mice	Intraperitoneal infection* 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
I	Mouse passage Stock virus (glycerinated)	Drug treated (8-10 g)					10/10	10/10	10/10	8/10
		Controls ” ”					10/10	10/10	10/10	8/10
II	Mouse passage Stock virus (fresh)	Drug treated (18-20 g)				10/10	10/10	10/10	6/10	
		Controls ” ”				10/10	10/10	8/10	5/10	
III	Guinea pig Virus (glycerinated)	Drug treated (14-18 g)	10/10	9/10	10/10	0/10				
		Controls ” ”	10/10	10/10	8/10	0/10				
IV	First return mouse Passage from guinea pig (glyc.)	Drug treated ” ”				9/10	10/10	9/10	9/10	
		Controls ” ”				10/10	10/10	10/10	7/10	
V	Tissue culture Virus (8th serial passage) (fresh)	Drug treated ” ”	9/10	4/10	0/10					
		Controls ” ”	8/10	1/10	0/10					
VI	First return mouse Passage from tissue culture (fresh)	Drug treated ” ”				10/10	10/10	10/10	10/10	
		Controls ” ”				10/10	10/10	10/10	10/10	
VII	First return mouse Passage from tissue culture (frozen)	Drug treated ” ”				10/10	10/10	10/10	10/10	
		Controls ” ”				10/10	10/10	10/10	10/10	

Note: Results are recorded as follows: Numerator = No. of mice paralyzed or dead. Denominator = No. of mice injected. The incubation periods were not significantly different between drug-treated and control animals. They varied in the different experiments between 3-10 days depending on the type and dilution of virus used.

* The infections dose was 0.1 cc of the indicated virus dilutions in Experiment I. In all subsequent experiments (II-VII) the dose was 0.06 cc.

dosage of drug employed for treatment. Thus, the percentage of drug-treated surviving animals hovered around 50% irrespective of whether the mice had previously been infected with approximately 1000 ID_{50} (10^{-4}) or 1 ID_{50} (10^{-7}). Again, there was no uniform improvement of the survival rate when the total amount of drug, administered in fractional daily doses over a period of 5 consecutive days, was stepped up from 40 mg to 80 mg. The following report deals with an unsuccessful attempt to verify the above results.

The drug (lot 7-8522 and lot 7-8564) was obtained through the courtesy of Lederle Laboratories, Inc. The drug was administered, 16 to 18 hours after intraperitoneal infection, by intraperitoneal injection of 4 daily divided doses as follows: 5 mg, 4 mg, 4 mg, 5 mg. The total amount of drug per diem was therefore 18 mg. This treatment was continued for 4 to 5 days. No symptoms of toxicity were observed with this dosage. Controls received 0.85% NaCl solution instead of drug. The mice were Rockland Swiss albino mice weighing, with one exception, from 14 to 20 g. The age of the animal is important because older mice are usually less susceptible by peripheral inoculation. Col SK murine virus was used in 3 forms: 1) mouse brain passage virus,)2 guinea pig brain virus and virus harvested from first return mouse passage, 3) tissue culture virus and virus harvested from first return mouse passage. One reason for choosing as infecting agent more than one source of virus was the fact that the 3 different strains—and their subpassages—represent stabile and unstabile forms of the same virus, with varying degrees of peripheral invasiveness,[2-4] which might conceivably respond selectively to the drug. Another more practical consideration was our desire to work under conditions identical with those employed by Sanders, SubbaRow, and Alexander, who operated with a seed virus obtained by passage of tissue culture virus to mice. In all other important details we followed closely the procedure described by these investigators. A total of 7 different experiments were run which are summarized in Table I.

From the data given in Table I it appears that phenosulfazole in adequate dosage had no demonstrable chemotherapeutic effect on the course of infection in mice induced with graded doses of Col SK virus under a wide variety of experimental conditions. It will further be noted that the drug not only failed to manifest any direct antiviral effects against the stabile forms of the virus (mouse passage virus, guinea pig virus, tissue culture virus), but likewise failed to display any indirect effects on the unstabile forms of the virus (first return mouse passage from infected guinea pig or tissue culture medium). In other words, there was nothing in our work to indicate that the drug has the power to cause reversion of the high peripheral infectivity of such viral forms to the low level of peripheral infectivity of their parent viral forms, a phenomenon which, if true, might have furnished a possible explanation for the curious observations reported by Sanders, SubbaRow, and Alexander. We are, therefore, at a loss to reconcile our own negative findings with the positive results reported by the earlier investigators.

Conclusions. Phenosulfazole had no demonstrable effect on the course of infection with murine SK poliomyelitis virus in mice.

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[2] Sanders, M., and Jungeblut, C. W., *J. Exp. Med.*, 1942, **75**, 631.

[3] Jungeblut, C. W., Feiner, R. R., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 31.

[4] Schultz, E. W., and White, S. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 266.

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16931

Production of Acute Pulmonary Edema by Ammonium Salts.[*]

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During the investigation of another problem,[1] in which guinea pigs were administered ammonium chloride by gavage, small overdoses of this chemical were found to result in pulmonary edema after a short interval.

In guinea pigs so treated, acute pulmonary edema appeared to be a consistent cause of death.[2,3] It was therefore considered worthwhile to experiment further with the thought of utilizing ammonium salts as a tool for a subsequent investigation of the pathogenesis of acute pulmonary edema.

Methods. Guinea pigs and white rats of both sexes were principally used. Six cats and 5 rabbits also were studied. Early in these studies, complete autopsies were done but when it became apparent that changes were limited to the chest, autopsy studies were restricted to the thoracic viscera.

Microscopic studies were carried out on all lungs in which the pathological diagnosis was in doubt, and also in a series of animals in which it was desired to study the histological changes of the grossly abnormal lungs. The lungs were fixed in one of 3 ways: by vascular perfusion[4] of 10% formalin in isotonic saline solution with 2.4% gum acacia added; by intratracheal injection of 10% formalin in isotonic saline solution; or by ligating and excising a pulmonary lobe, immersing it in 10% formalin in isotonic saline, and agitating it constantly on a shaking device for 24-48 hours. Immersion fixation of an entire lobe gave the best results for the demonstration of edema exudate. All lung sections were stained with hematoxylin and eosin.

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[*] This work was aided by a grant from the Baxter Laboratories, Inc., administered by Professor W. F. Windle.

[†] Presented to the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] Windle, W. F., Koenig, H., and Jensen, A. V., *Arch. Neurol. and Psychiat.*, 1946, **56**, 428.

[2] Koenig, H., and Koenig, R., *Fed. Proc.*, 1948, **7**, 67.

[3] Koenig, H., and Koenig, R., *Anat. Rec.*, 1948, **100**, 52.

A 6% solution of ammonium chloride in water (weight in volume) was used at all times with the exception of the animals administered other salts of ammonium to determine the specificity of the cation with regard to the lung changes. Control animals were treated like the experimental animals except for the administration of ammonium chloride. These animals were also anesthetized with pentobarbital ("Veterinary Nembutal") just before removal of the lungs. Further details of procedure will be given in the appropriate sections to follow.

Results. A. Dosage and routes of administration. Guinea pig. Most of our experience has been with this animal. We have seen typical pulmonary alterations with ammonium chloride given by the gavage, subcutaneous, intramuscular, intraperitoneal and intravenous routes of administration. The dose of ammonium chloride depended upon its mode of introduction. The slower the rate of absorption, the larger the dose had to be. Thus, a dose which would produce acute pulmonary edema by gavage would, when injected into the peritoneal cavity, usually kill the animal before sufficient time had elapsed for the evolution of these changes. Ammonium chloride could be successfully injected into the vein only by slow drip. Another factor of importance was the weight of the animal; the usual direct relationship between body weight and dosage appeared to apply.

The gavage and intraperitoneal routes were utilized almost exclusively because the results were more dependable. By stomach tube, the effective dose was 0.09 to 0.12 g/100 g of body weight; intraperitoneally, it was 0.05 to 0.07 g/100 g of body weight. Thirty of the 40 experimental guinea pigs succumbed to acute pulmonary edema. Wherever lung changes failed to appear, it could be attributed to one of two causes. If the amount of ammonium chloride was too great, or if it was too rapidly absorbed, death resulted from cardiac failure, or more frequently from respiratory failure, before the appearance of lung changes. On the other hand, if the dose was inadequate, or if absorption appeared retarded, the lung changes were not seen and other manifestations of toxicity were often completely absent. Our experience suggested that a definite concentration of the ammonium ion has to be attained and maintained in the blood for a period of at least 10 or 15 minutes for the development of pulmonary edema.

Rat. Ammonium chloride solution was administered to this animal by stomach tube and by intraperitoneal injection. The latter route proved more convenient and reliable. The effective dose for the intraperitoneal route was 0.04 g/100 g (0.66 cc of a 6% solution). Administration resulted in acute pulmonary edema in all of 10 rats.

Cat. The cat proved to be more resistant to the pulmonary edema produced by ammonium chloride than the guinea pig or the rat. Vomiting and salivation were a common occurrence with toxic doses of ammonium chloride given intraperitoneally or by gavage, but care was taken to prevent aspiration. In later experiments, the trachea was cannulated following local infiltration with 1% procaine in order to circumvent these difficulties. Of the 6 cats used, pulmonary edema occurred in 3 animals. The animals in the latter group weighed 1950-2750 g each. Two were given 35 cc of a 6% solution of ammonium chloride intraperitoneally. A third was given 69 cc of a 6% solution of ammonium chloride intraperitoneally in 4 divided doses over a period of 2 hours.

Rabbit. In none of 5 animals did acute pulmonary edema occur after ammonium chloride was given intraperitoneally or by gavage in doses great enough to cause toxic symptoms culminating in death.

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[4] Koenig, H., Groat, R. A., and Windle, W. F., *Stain Technology*, 1945, **20**, 13.

B. Specificity of the ammonium ion. In order to determine the specificity of the ammonium moiety of ammonium chloride in this phenomenon, 6 guinea pigs, each weighing about 350 g, were used. One was given 6 cc of 20% ammonium nitrate, a second was given 7 cc of 6.4% ammonium acetate, a third 7 cc of 10% ammonium bromide, a fourth 7 cc of 6% ammonium chloride, and a fifth 7 cc of 7.4% ammonium sulfate. The 4 latter solutions were roughly equivalent with respect to the concentration of ammonium. A sixth animal was given 7 cc of water to serve as a control. Gavage administration was used in these experiments. The first 5 animals all succumbed to acute pulmonary edema. The lungs of the control were normal.

Guinea pigs given the standard dose of ammonium chloride by gavage showed a blood pH of 6.8 to 7.2, 15 to 30 minutes later. Two mechanisms are involved in the production of this acidosis, acid hydrolysis of ammonium salts, and the conversion of ammonia into urea. The first mechanism does not operate with an ammonium salt of a weak acid, such as ammonium acetate. However, guinea pigs and cats rendered even more severely acidotic by gavage administration of a sodium lactate buffer, or a dilute solution of hydrochloric acid, never showed pulmonary edema. It is evident, therefore, that the ammonium ion rather than the acidosis is the primary factor in the pathogenesis of this form of lung edema.

C. Syndrome of ammonium intoxication. In the guinea pig and rat, the characteristic sequence of events transpired in 15 to 60 minutes, taking less than 30 minutes in most of the animals. A considerable increase in rate and depth of respiration occurred several minutes after the administration of ammonium chloride. Weakness and difficulty in locomotion soon appeared; at about the same time, hyperexcitability for tactile, auditory and painful stimuli was noted. Shortly thereafter, muscle fasciculations appeared over most of the body and could be best seen in the tongue. These usually preceded the onset of one or more generalized tonic convulsions. Occasionally the convulsions appeared before the fasciculations. Coma quickly supervened, the corneal reflex was absent and no stimulus however great would bring about a response. The respirations were by this time greatly reduced in frequency and were deep and gasping in character. Stridor was frequently heard. A serosanguineous, or rarely a serous, fluid exuded from the nostrils or mouth before death. Occasionally, no fasciculations or convulsions occurred, or perhaps only a terminal convulsion; yet acute pulmonary edema was present at autopsy.

The above description held also for the cat, with several exceptions. The dose required was greater and the time interval before lung edema appeared was longer, varying from one to 3 hours. Sialorrhea usually occurred and vomiting was common. This animal appeared to be on the whole more resistant to ammonium pulmonary edema than were the rodents. Another notable difference was the character of the edema fluid issuing from the upper respiratory tract. In the cat, the fluid was never blood-tinged, whereas, in the rat and guinea pig, blood-tinged edema fluid was the rule, with very few exceptions.

The effects of toxic doses of ammonium would appear to be chiefly on the central nervous system, at least until pulmonary edema supervenes. They were manifested by muscle fasciculations, tonic convulsions and

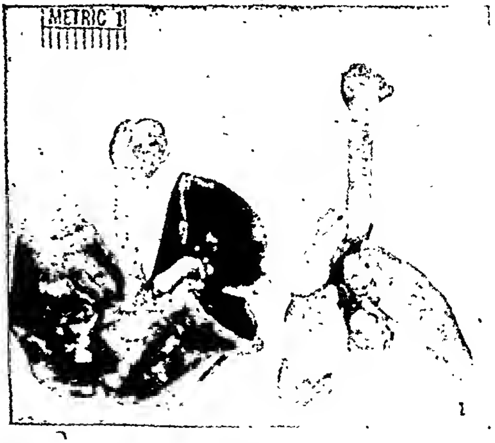
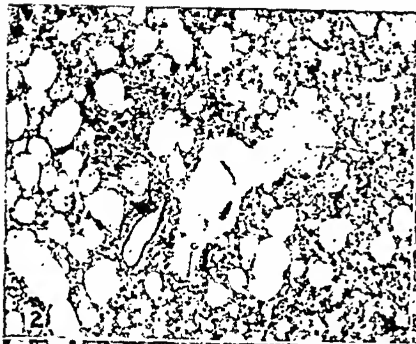
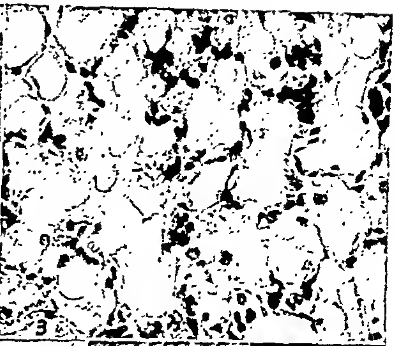
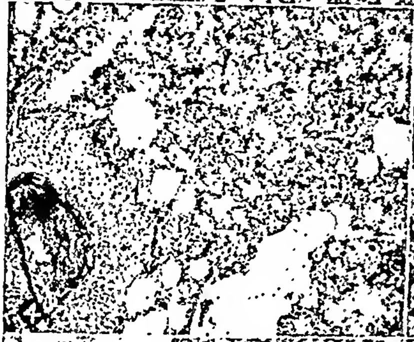


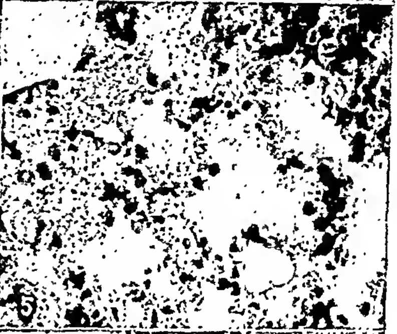
FIG. 1.

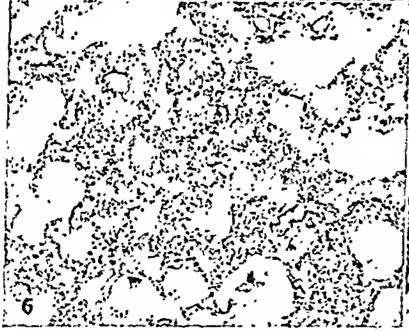
Photograph of gross specimens. On the left are the trachea and lungs from a rat that died 20 minutes after an intraperitoneal injection of ammonium chloride. The lungs are swollen and present pleural surfaces which are discolored by areas of hemorrhage and congestion. At the right are the trachea and lungs from a control rat.











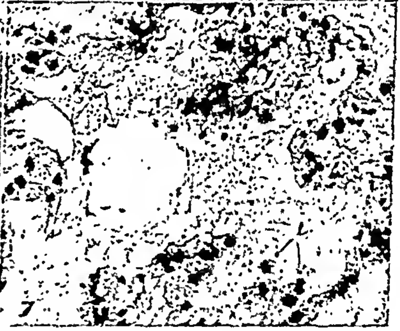


FIG. 2. Photomicrograph of a typical area from a section of lung from a guinea pig which succumbed to acute pulmonary edema 30 minutes after gavage administration of ammonium chloride. Many of the alveoli are filled with a homogeneous precipitate of edema fluid (e). × 64.

FIG. 3. Photomicrograph, at higher magnification, of part of the area shown in Fig. 2 illustrating the homogeneous edema exudate which contains many air bubbles. Hemorrhage is not a prominent feature here, but occasional small groups of extravasated red blood cells are present in the alveoli. × 263.

FIG. 4. Photomicrograph illustrating a typical area of a section of lung from rat which died 25 minutes after an intraperitoneal injection of ammonium chloride. Most of the alveoli in this field are filled with a homogeneous eosinophilic exudate in which air bubbles and occasional blood cells are included. A branch of the pulmonary artery in the lower left hand field is surrounded by a wide "collar" of edema exudate containing many red blood cells. × 64.

FIG. 5. Photomicrograph at higher magnification of a portion of the field shown in Fig. 4. The edema exudate and the dilatation and intense congestion of the alveolar capillaries are prominent features. × 263.

FIG. 6. Photomicrograph of a section taken from the lung of a cat which died of acute pulmonary edema 2½ hours after the intraperitoneal injection of ammonium chloride. A fibrillar exudate fills many of the alveoli. A number of small veins are seen dilated and filled with blood cells. × 64.

FIG. 7. Photomicrograph at higher magnification of part of a single alveolus in the field shown in Fig. 6. Many fibrillar strands are in the edma exudate. A small bubble (a) of air still remains in the center of the alveolus, another smaller one is at the side. Occasional macrophages (septal cells(s)), and leucocytes (l) appear in what was formerly the alveolar space. Capillaries in the alveolar septa are turgid with blood cells. × 263.

dyspnea. The fasciculations suggested repetitive discharging of primary motor neurons of the brain stem and spinal cord. The tonic convulsions indicated a greatly heightened reflex excitability of the spinal cord. The dyspnea which occurred early had a two-fold origin. Acidosis was an important factor, which probably stimulated respiration reflexly through the chemoreceptors[5] and through an increase in CO_2 tension in the blood which resulted from a depression in pH. In addition, ammonium is a direct respiratory stimulant;[6] we noted dyspnea with ammonium acetate, a salt which produces no immediate depression of blood pH.

D. Anatomical changes. Gross pathology found at necropsy was observed only in the chest and upper respiratory tract. The nasal cavity, larynx, trachea and bronchi were filled with a frothy fluid, usually blood-tinged in the guinea pig and rat, but serous in the cat. These passageways were otherwise unobstructed. Pleural fluid was never seen. The lungs were increased in size (Fig. 1), collapse being prevented by the fluid in the lung parenchyma. The pleural surface of the lungs was always discolored; in some animals there was only a petechial distribution of deep red spots, in others a massive hemorrhagic involvement, but an appearance intermediate between these extremes was more common. The changes showed no predilection for lobes or parts of lobes. On section, the lung was deep red in many places and a frothy serosanguineous fluid could be readily expressed. Congestion of blood vessels and parenchyma was marked. The right atrium, right ventricle and the venae cavae entering the heart were usually moderately dilated, but the left ventricle and left atrium appeared normal.

Histological alterations were limited to the lung and the brain.[‡] By far the most important change was the massive pulmonary edema with variable degrees of hemorrhage. The alveolar spaces and smaller air passages were filled with a precipitated pale eosinophilic material in which air bubbles and red blood cells were often included. This edema exudate was usually homogeneous in the guinea pig (Figs. 2 and 3) and rat (Figs. 4 and 5) when the lung was fixed by total immersion, but was distinctly fibrillar in the cat. Interstitial edema was not a prominent finding except in the rat where periarterial "collaring" or "cuffing" of edema exudate was always present. Hemorrhage into the alveoli and the interstitial tissues was always present, but varied greatly in extent and degree. Vascular congestion was intense and involved not only the larger vessels, but also the capillaries. In the cat (Fig. 6 and 7) it was not unusual to find alveolar capillaries that were seven or eight red blood cell diameters in width and filled with densely packed blood cells.

Discussion. The lung changes produced by toxic doses of ammonium salts appear to have escaped notice. Underhill and Kapsinow[7] determined the minimum lethal dose for a series of ammonium salts in white rats, producing death within three hours. They concluded that the toxicity of these compounds, with a few exceptions, varied directly with the ammonium content of the compound. However, these investigators did not report the presence of pulmonary edema, nor did they state the cause of death in the animals studied. Chenoweth and his colleagues[8] studied the toxicity of ammonium gluconate which was given intravenously to cats, but they did not record the presence of pulmonary edema at autopsy.

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[5] Schmidt, C. F., Macleod's Physiology in Modern Medicine, Philip Bard, C. V. Mosby Co., St. Louis, 9th edition, 1941, 561, 567.

[6] Brassfield, C. R., Hansen, E. T., and Gesell, R., *Fed. Proc.*, 1946, 5, 10.

[‡] Pathological changes have been seen in the brain and are described elsewhere.[1]

[7] Underhill, F. P., and Kapsinow, R., *J. Biol. Chem.*, 1922, 54, 451.

[8] Chenoweth, M. B., Civin, H., Salzman, C., Cohen, M., and Gold, H., *J. Lab. and Clin. Med.*, 1941, 26, 1574.

It is in the clinical literature that one finds a hint of this toxic effect of ammonium salts. Fazekas[9] described 2 cases of suicide resulting from the ingestion of ammonium chloride. One woman died 10 hours afterward and autopsy revealed, among other findings, an advanced pulmonary edema. The brain changes which were described were characteristic of anoxia. The amount of ammonium chloride ingested was unknown.

Abnormal increases of ammonium in the blood are occasionally encountered in disease. McNeil and Levy[10] found that this occurred most often in conjunction with acute disease processes involving the liver. In view of the important role the liver normally plays in the transformation of ammonium to urea, this is not surprising. While there is apparently little danger of ammonium intoxication with the usual therapeutic doses of ammonium salts, the possibility exists that large doses, such as are occasionally given to cardiac patients to promote diuresis (up to 40 g a day)[11] in the presence of an hepatic insufficiency, may result in a blood concentration of ammonium great enough to cause toxic manifestations—even pulmonary edema. Such an occurrence would ordinarily be imputed to the cardiac disorder rather than the ammonium.

Summary. (1) Ammonium salts in toxic doses produced an acute pulmonary edema in the rat, guinea pig and cat. The cat was more refractory to this effect than the rat and guinea pig. The rabbit did not appear to be susceptible to ammonium lung edema. (2) While a number of routes of administration of ammonium chloride were found effective for the production of acute pulmonary edema, the gavage route (0.09-0.15 g per 100 g of body weight) was most dependable for the guinea pig, and the intraperitoneal route (0.04 g per 100 g of body weight) for the rat. (3) The ammonium ion was found to be the agent responsible for the lung edema. Many ammonium salts produced this effect. Acidosis was ruled out as the primary factor, because acidosis produced in other ways did not result in lung edema. (4) The syndrome of ammonium intoxication consisted chiefly of dyspnea, muscle fasciculations and convulsions, terminating in an early acute pulmonary edema. (5) Gross changes at autopsy were limited to the thoracic cavity. They consisted of pulmonary edema and congestion with variable degrees of hemorrhage and moderate dilatation of the right ventricle, right atrium, and entering venae cavae. The microscopic appearance of the lungs was one of edema, congestion and hemorrhage.

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[9] v. Fazekas, G. I., *Deutsch. Ztschrft. f.d. ges. gericht. Med.*, 1934, **23**, 225.

[10] McNeil, H. L., and Levy, M. D., *J. Lab. and Clin. Med.*, 1917, **3**, 18.

[11] Keith, N., quoted by Sollmann, T., A Manual of Pharmacology, W. B. Saunders, Co., 7th edition, 1948, 776.

16932

Changes in Blood Chemistry in Rats Following Electrically-Induced Seizures.*

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A maximal seizure consists of violent neural and muscular activity lasting many seconds and might be expected to involve considerable changes in brain and blood chemistry. Klein and Olsen[1,2] found that 10 seconds of convulsive activity accelerates the glucose metabolism of brain and results in an approximately two-fold increase in brain lactate and a decrease in high-energy phosphate bonds. McLaughlin and Hurst[3] observed moderate decreases in plasma pH and alkali reserve and corresponding increases in blood lactate, and others[4-7] have found increases in plasma phosphate following idiopathic convulsions in humans. The post-convulsive depression and other sequelae of seizures may be the result of metabolic activity occurring during seizures. In order to determine the magnitude and duration of chemical changes occurring in a commonly used experimental animal, the work presented here was undertaken.

Methods. Adult male rats of the Sprague-Dawley strain were used. Maximal seizures were induced by passing a 150 mA current of 0.2 secs duration between Spiegel corneal electrodes.[8] Blood was obtained by heart puncture, and heparin was used as an anticoagulant. Plasma was separated anaerobically by the method of Davenport.[9] Its pH was determined by means of a standardized glass electrode, and its carbon dioxide by the method of Van Slyke and Neill.[10] Plasma lactate was determined by the method of Barker and Summerson,[11] and plasma inorganic phosphate was determined by the method of Fiske and SubbaRow.[12] Plasma sodium and potassium were estimated by means of a Perkin-Elmer flame photometer (Model 52A) with internal lithium standard.

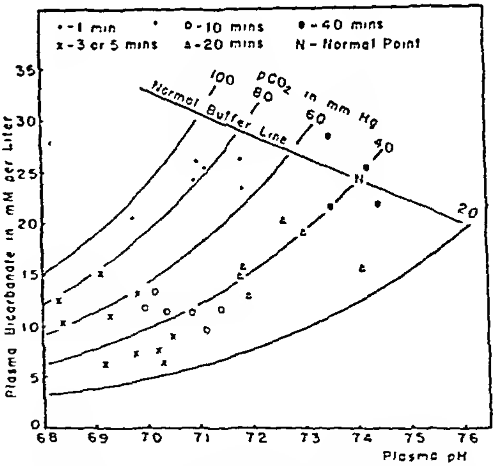

FIG. 1.

pH-bicarbonate diagram showing blood acid-base changes following maximal seizures in rats.

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* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

† Fellow of the University of Utah Research Fund.

[1] Klein, J. R., and Olsen, N. S., *J. Biol. Chem.*, 1947, **167**, 747.

[2] Olsen, N. S., and Klein, J. R., *Proc. A. Res. Nerv. and Ment. Dis.*, 1946, **26**, 118.

[3] McLaughlin, F. L., and Hurst, R. H., *Quart. J. Med.*, 1933, **2**, 419.

[4] Goldstein, H., and McFarland, R. A., *Am. J. Psych.*, 1939, **96**, 771.

[5] del Roncal, F. P., *J. Nerv. and Ment. Dis.*, 1941, **94**, 133.

[6] Weil, A., and Liebert, E., *Arch. Neur. and Psych.*, 1937, **37**, 584.

[7] Hirschfelder, A. D., and Haury, V. G., *Arch. Neur. and Psych.*, 1938, **40**, 66.

[8] Toman, J. E. P., Swinyard, E. A., and Goodman, L. S., *J. Neurophysiol.*, 1946, **9**, 231.

[9] Davenport, H. W., *Science*, 1947, **105**, 2717.

[10] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[11] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

[12] Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

which was given intravenously to cats, but they did not record the presence of pulmonary edema at autopsy.

It is in the clinical literature that one finds a hint of this toxic effect of ammonium salts. Fazekas[9] described 2 cases of suicide resulting from the ingestion of ammonium chloride. One woman died 10 hours afterward and autopsy revealed, among other findings, an advanced pulmonary edema. The brain changes which were described were characteristic of anoxia. The amount of ammonium chloride ingested was unknown.

Abnormal increases of ammonium in the blood are occasionally encountered in disease. McNeil and Levy[10] found that this occurred most often in conjunction with acute disease processes involving the liver. In view of the important role the liver normally plays in the transformation of ammonium to urea, this is not surprising. While there is apparently little danger of ammonium intoxication with the usual therapeutic doses of ammonium salts, the possibility exists that large doses, such as are occasionally given to cardiac patients to promote diuresis (up to 40 g a day)[11] in the presence of an hepatic insufficiency, may result in a blood concentration of ammonium great enough to cause toxic manifestations—even pulmonary edema. Such an occurrence would ordinarily be imputed to the cardiac disorder rather than the ammonium.

Summary. (1) Ammonium salts in toxic doses produced an acute pulmonary edema in the rat, guinea pig and cat. The cat was more refractory to this effect than the rat and guinea pig. The rabbit did not appear to be susceptible to ammonium lung edema. (2) While a number of routes of administration of ammonium chloride were found effective for the production of acute pulmonary edema, the gavage route (0.09-0.15 g per 100 g of body weight) was most dependable for the guinea pig, and the intraperitoneal route (0.04 g per 100 g of body weight) for the rat. (3) The ammonium ion was found to be the agent responsible for the lung edema. Many ammonium salts produced this effect. Acidosis was ruled out as the primary factor, because acidosis produced in other ways did not result in lung edema. (4) The syndrome of ammonium intoxication consisted chiefly of dyspnea, muscle fasciculations and convulsions, terminating in an early acute pulmonary edema. (5) Gross changes at autopsy were limited to the thoracic cavity. They consisted of pulmonary edema and congestion with variable degrees of hemorrhage and moderate dilatation of the right ventricle, right atrium, and entering venae cavae. The microscopic appearance of the lungs was one of edema, congestion and hemorrhage.

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[9] v. Fazekas, G. I., *Deutsch. Ztschrft. f.d. ges. gericht. Med.*, 1934, **23**, 225.

[10] McNeil, H. L., and Levy, M. D., *J. Lab. and Clin. Med., 1917, 3, 18.*

[11] Keith, N., quoted by Sollmann, T., A Manual of Pharmacology, W. B. Saunders, Co., 7th edition, 1948, 776.

16933

Application of the Transducer Tube to the Recording of the Peripheral Pulse.

Howard J. Curtis* and John L. Nickerson.

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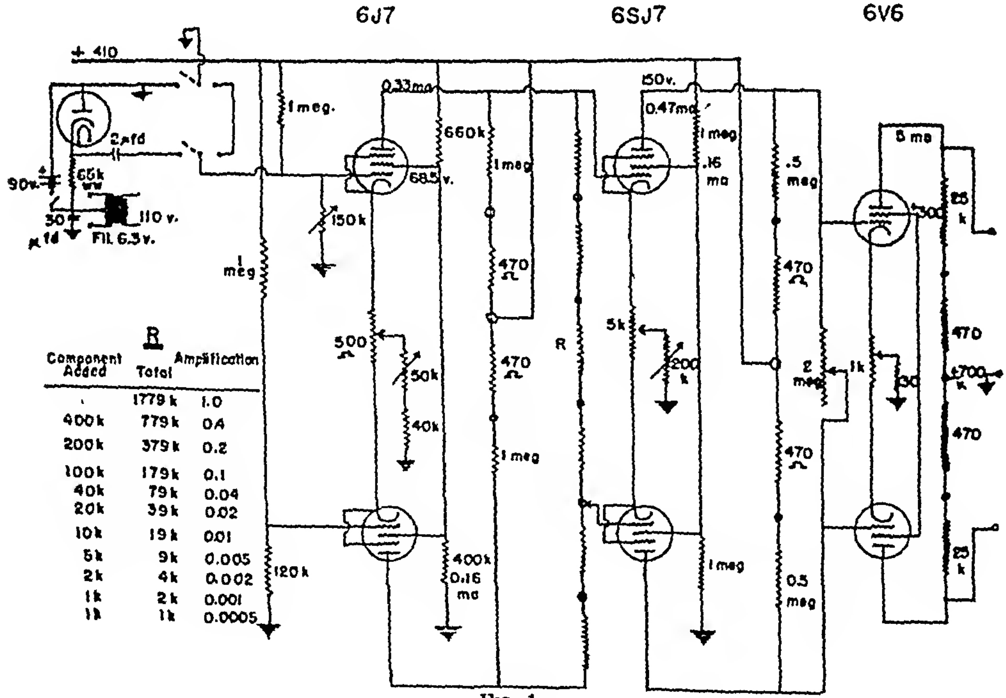
The observation and recording of the form of the venous, radial or other peripheral pulses in man has some value in teaching the dynamics of the circulation as well as in aiding clinical diagnosis.[1] Conventionally the pulsation is picked up by a cup-like device pressed over the area involved. The impulses are transmitted by rubber tubing to a tambour or segment capsule for recording.

This paper presents the application of the Transducer Tube (RCA Developmental Tube No. C 798 B) to this problem. In this tube the plate is connected to a peg extending through a flexible diaphragm to the outside so that micromovements of the peg will produce changes in the plate current. A light weight button and lever are attached to the peg. The button is touched to the pulsating area and its movements are transformed to current changes, amplified and recorded by an ink writing crystograph (Fig. 1) or by a galvanometer recording on photographic paper. The natural frequency of the moving part is remarkably high and with careful mechanical design and a light weight button it can be as high as 100 cycles per second, a value which


6J7
6SJ7
6V6
+ 410
2 μfd
90v.
65k ww
30 μfd
110 v.
Fil. 6.3v.
1 meg.
1 meg
150k
0.33 ma
660k
68.5 v.
1 meg
470 Ω
500 Ω
50k
40k
470 Ω
1 meg
120 k
400k
0.16 ma
R
150v.
0.47 ma
1 meg
.16 ma
5k
200 k
.5 meg
470 Ω
2 meg
470 Ω
1 meg
0.5 meg
5 ma
300
25 k
1k
300
470
700 k
470
25 k

Component Added	R Total	Amplification
	1779 k	1.0
400k	779k	0.4
200k	379k	0.2
100k	179k	0.1
40k	79k	0.04
20k	39k	0.02
10k	19k	0.01
5k	9k	0.005
2k	4k	0.002
1k	2k	0.001
1k	1k	0.0005

Fig. 1.

The transducer circuit with its amplifier of successive push-pull stages of 6J7, 6SJ7, and 6V6 to the direct writer is shown.

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* Now at the Department of Physiology, Vanderbilt University, Nashville, Tenn.

[1] Wiggers, C. J., Physiology in Health and Disease.

TABLE I.

Concentrations of Electrolytes in Rat Plasma Following Maximal Electroshock Convulsions.

Time after convulsions, min.	Lactate, mEq/l	Phosphate, mM/l	Sodium, mEq/l	Potassium, mEq/l
Control	3.1 ± 0.2* (12)	1.9 ± 0.1 (8)	142.5 ± 0.1 (12)	4.8 ± 0.2 (12)
1	20.8 ± 1.9 (6)	3.4 ± 0.3 (6)	155.1 ± 1.1 (6)	5.1 ± 0.1 (6)
5	20.3 ± 1.5 (6)	2.8 ± 0.3 (6)	151.1 ± 0.6 (6)	4.4 ± 0.1 (6)
10	—	—	147.2 ± 0.2 (6)	4.0 ± 0.2 (6)
20	11.1 ± 1.6 (6)	2.5 ± 0.1 (6)	147.3 ± 1.1 (6)	3.8 ± 0.2 (6)
40	3.9 ± 0.5 (6)	2.1 ± 0.1 (6)	140.6 ± 0.4 (5)	4.6 ± 0.1 (5)
60	3.7 ± 0.6 (6)	1.8 ± 0.1 (6)	—	—

* Standard error of the mean.

Results. The plasma pH and bicarbonate concentration, observed at intervals after seizures, are plotted on the pH-bicarbonate diagram shown in Fig. 1. The plasma lactate, phosphate, sodium and potassium concentrations are given in Table I.

One minute after a maximal seizure, respiratory acidosis was present, the pCO_2 averaging 80 mm Hg. There was also a severe metabolic acidosis. Total extra fixed-acids of the blood were greater than 20 mEq/l, and the sodium concentration had risen 12 mEq/l. At the end of 5 minutes the respiratory acidosis had disappeared; but the metabolic acidosis was still extreme, and all observed pH values were below 7.05. Thereafter the metabolic acidosis became progressively less severe until recovery was attained at 40 minutes. Simultaneously the plasma sodium concentration returned to normal, and the plasma potassium concentration fell slightly below normal.

Discussion. The metabolic changes produced by a maximal seizure can be explained by the vigorous muscular activity and cessation of respiration occurring during the convulsion. The acidosis is as severe as that observed in completely uncontrolled diabetes mellitus, and the rise in sodium concentration is equal to that produced by overdosage with desoxycorticosterone acetate.[13] It is likely that the metabolic changes, rather than purely electrical events, in the brain, account for most of the post-seizure depression whose time course parallels that of the chemical changes in the blood.[8] Extreme metabolic acidosis, produced by ammonium chloride, itself reduces the excitability of the brain as measured by an increase in electroshock threshold.[14] A rise in plasma sodium concentration is similarly associated with reduced cerebral excitability.[13] If similar changes occur in humans undergoing electroshock therapy of psychotic disorders, it would appear important to investigate the relative contributions of the changes in brain and blood chemistry and the electroshock itself to the salutary results of the treatment.

Summary. Severe respiratory and metabolic acidosis, accompanied by increases in plasma lactate, phosphate and sodium concentrations, occur in rats following maximal electrically-induced seizures. Recovery is complete in 40 minutes. It is suggested that the profound chemical changes occurring during the seizure may contribute to the post-ictal depression.

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[13] Woodbury, D. M., and Davenport, V. D., in press.

[14] Hendley, C. D., Davenport, H. W., and Toman, J. E. P., *Am. J. Physiol.*, 1948, **153**, 580.

TABLE I.
Recovery of C^{14} Activity in the Fatty Acids Isolated from Organs and Tissues 24 Hours After the Injection of C^{14}-Palmitic Acid as Tripalmitin.

Tissues	Rat 1 (188 g)	Rat 2 (152 g)	Rat 3 (161 g)	Rat 4 (159 g)
	% of the injected C^{14} recovered			
Liver	2.64	5.68	3.84	3.31
Lungs	0.58	2.58	0.24	0.14
Spleen	0.51	1.01	0.10	0.26
Kidney	0.42	0.33	—	—
Small Intestine*	1.33	1.54	0.95	0.79
Large Intestine*	—	—	0.13†	0.13†
Brain	—	—	0.05	0.05
Depot:				
Perirenal	0.30	0.26	—	—
Genital	0.31	0.34	—	—
Omental	0.21	0.09	—	—
Mesenteric	0.74	0.39	—	—
Skin‡	6.6	3.2	—	—
Carcass§	5.6	6.6	15.9	13.8
Feces	0.76‖	1.13‖	0.36	0.32
Total	19.8	22.1	21.6	18.8

* Washed free of intestinal contents as described in text.
† The large intestines of rats 3 and 4 were pooled; 0.13 is half of the total C^{14} recovered.
‡ Includes hair and subcutaneous fat
§ Includes all organs and tissues not specified above.
‖ The value includes the activity of the feces plus that of the large intestine and its contents.

utilized, for not only did they find weight improvement and nitrogen retention in 2 dogs, but they were also unable to find any considerable portion of the infused fat stored in an unmodified form. A gain in body weight in dogs that received fat emulsions intravenously was also noted by Meng and Freeman,[2] but they point out that such results furnish no direct proof of fat utilization.

In order to study the path of utilization of fat introduced by the intravenous route, palmitic acid labeled at its 6th carbon with C^{14} was used in the present investigation. It is shown here that palmitic acid, when injected directly into the blood stream in the form of highly emulsified tripalmitin, is oxidized at a rapid rate, converted to phospholipid, and deposited in the fat depots.

Experimental. Preparation of emulsion. Palmitic acid containing C^{14} in the sixth carbon atom was synthesized, as described in an earlier communication,[5] and then esterified with glycerol by a modification of the method of Feuge *et al.*[6] The labeled tripalmitin was melted, together with half its weight of glycerol monostearate, and enough 5% glucose solution was added to yield an emulsion containing 4% fat. A propeller attached to a high speed motor was then inserted and the mixture was agitated violently for 5 minutes at a temperature maintained near the boiling point. The resulting emulsion was irradiated with supersonic energy (Ultra-sonorator, Model SL520) for 10 minutes at 700,000 cycles per second. The usual volume so treated was 3-5 ml. The particles obtained were almost all less than 1 μ in diameter.

Treatment of animals and collection of expired $C^{14}O_2$. Rats of the Long-Evans strain, weighing from 152 to 188 g, were used. After being fasted for 24 hours, they were anesthetized lightly with ether. One or 1.5 ml of the emulsion containing 20-25 mg of labeled tripalmitin (from 4 to 7 × 10^4 counts per minute per mg tripalmitin) were then slowly injected into the foot vein of each rat. The injection lasted about one minute. The rats were then placed in large pyrex desiccators arranged so that urine and feces could be collected and CO_2-free air could be drawn

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[5] Dauben, W. G., *J. Am. Chem. Soc.*, 1948, **70**, 1376.

[6] Feuge, R. O., Kraemer, E. A., and Bailey, A. E., *Oil and Soap*, 1945, **22**, 202.

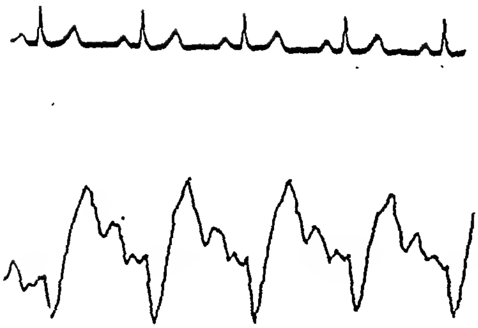

FIG. 2.
Illustrates the simultaneous record of the jugular pulse and the electrocardiogram.

compares favorably with the best devices in the literature. In Fig. 2 is shown a record of the venous pulse with a simultaneous recording of the electrocardiogram.

In presenting this application of the transducer tube it is desired to point out that this is only one of its many possible uses and that it should have rather wide usefulness in biology and medicine. The tube itself is about the size of the end of a pencil and can thus be used in small places if necessary. Furthermore, since the plate current in the transducer tube depends upon the displacement of the plate it is possible with a slight change in the circuit of Fig. 1 to measure displacements rather than changes in displacement. The sensitivity is high, and it has been our experience that it is easier to obtain a given sensitivity with a transducer than with a resistance wire strain gage.

Summary. This paper presents the application of the transducer tube to the detection and recording of movements of the type associated with the peripheral venous pulses. An adequate amplifier circuit is given and the usefulness of the device in other applications is suggested.

16934

The Fate of C^{14}-Labeled Palmitic Acid Administered Intravenously as a Tripalmitin Emulsion.*†

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From the Division of Physiology of the Medical School and the Department of Chemistry, University of California, Berkeley.

The need for providing sufficient calories in small volumes for parenteral feeding has focussed attention upon methods for the intravenous administration of fat in a highly emulsified form. Now that several investigators have shown that the intravenous administration of fat can be achieved with some degree of safety,[1-3] it has become of importance to determine whether fat so administered pursues a normal metabolic path. Several attempts have been made to study the utilization of parenterally administered fats but the results obtained until recently were not conclusive. Thus Dunham and Brunschwig[4] failed to observe protein-sparing effects in 9 of 11 dogs when a highly emulsified fat was injected intravenously for periods as long as one month. Their emulsions, however, were quite toxic. McKibbin *et al.*,[1] on the other hand, have concluded that intravenously administered emulsions of fat are

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* Aided by grants from the American Cancer Society (recommended by the Committee on Growth), and the Cutter Laboratories, Berkeley.

† A preliminary report of the results of the present investigation has appeared in *Science.*

[1] McKibbin, J. M., Pope, A., Thayer, S., Ferry, R. M., Jr., and Stare, F. J., *J. Lab. Clin. Med.*, 1945, **30**, 488.

[2] Meng, H. C., and Freeman, S., *J. Lab. Clin. Med.*, 1948, **33**, 689.

[3] Shafiroff, B. G. P., Baron, H., and Roth, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 387.

[4] Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, **48**, 395.

TABLE II.
Incorporation of Labeled Fatty Acids into Phospholipid and Nonphospholipid Fractions of Tissues 24 Hours After the Injection of C^{14}-Palmitic Acid as Tripalmitin.

Tissue	Lipid fractions	Rat 1	Rat 2	Rat 3	Rat 4
		% of each tissue's lipid C^{14} found in respective fraction			
Liver	Phospholipid fatty acids	34	50	72	72
	Nonphospholipid "	66	50	28	28
Small Intestine	Phospholipid "	64	71	74	78
	Nonphospholipid "	36	29	26	22
Large Intestine	Phospholipid "	—	—	69*	69*
	Nonphospholipid "	—	—	31*	31*

* The large intestines of rats 3 and 4 were pooled and the value shown is half of the total.

their livers, 34 and 50%, respectively, of their fatty acids were in the phospholipid fraction.

Comment. The manner in which long-chain fatty acids are utilized when administered by routes other than the gastrointestinal tract has aroused considerable interest in recent years. The use of radioactive carbon provided, for the first time, a direct method for determining the rate at which an animal can convert parenterally administered, long-chain fatty acids to CO_2. In the present investigation, a 16-carbon fatty acid containing C^{14} in the sixth carbon atom was used and injected in the form of its triglyceride. It is shown here that as much as 59% of the sixth carbon atoms are converted to CO_2 in 24 hours. This observation, in addition to the demonstration of storage of significant amounts of the labeled fatty acids in adipose tissues and their recovery in the phospholipids isolated from liver and intestine, can leave no further doubt that palmitic acid is utilized when used for intravenous feeding.

Since the completion of the present study, Geyer *et al.* have reported on the oxidation of intravenously administered trilaurin.[8] They injected a fat emulsion containing trilaurin in which the carboxyl group of the 12-carbon fatty acid was labeled with C^{14}, and found that in 4.5 hours, 71% of the C^{14} injected into rats was exhaled as CO_2. In this interval we found that only 11-23% of the sixth carbon atoms of palmitic acid, which had been administered intravenously as a tripalmitin emulsion, are exhaled as CO_2. The more rapid oxidation rate of trilaurin is most likely due to the fact that the 12-carbon fatty acid is not deposited in adipose tissue to a measurable extent.

Summary. 1. In order to test the extent of utilization of fat emulsions in parenteral feeding, palmitic acid labeled with C^{14} at its sixth carbon was injected intravenously, in the form of the triglyceride, into fasted rats.

2. From 36 to 59% of the administered C^{14} was expired as CO_2 in 24 hours.

3. Considerable amounts of the administered fatty acids were stored in adipose tissues throughout the body.

4. The availability of intravenously administered tripalmitin for metabolic purposes is further shown by the finding that as much as 78% of the radioactive fatty acids recovered from the liver and small intestine had been incorporated into phospholipids.

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[8] Geyer, R. P., Chipman, J., and Stare, F. J., *J. Biol. Chem.*, 1948, **176**, 1469.

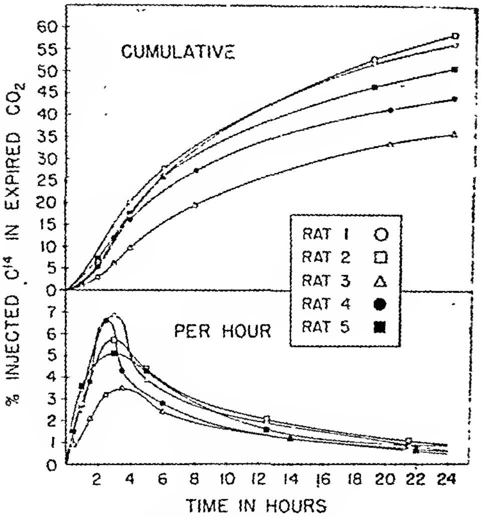

Fig. 1.
Rate of conversion of intravenously injected C^{14}-labeled tripalmitin to CO_2.

through. Water, but not food, was provided. The air leaving the desiccator was dispersed, by a sintered glass disc, into a tower containing a sodium hydroxide solution. The sodium hydroxide was changed at intervals so that collection times were 0-2, 2-4, 4-6, 6-19, and 19-24 hours for three rats, and 0-1, 1-2, 2-3, 3-4, 4-8, 8-20, and 20-24 hours for 2 rats.

The expired CO_2 collected as the carbonate was precipitated as $BaCO_3$ and prepared for counting as described previously.[7]

Treatment of tissues. At the end of 24 hours, the rats were deeply anesthetized with ether. The organs and tissues indicated in Table I were removed and covered with alcohol. The small and large intestine were washed free of their contents with cold ethyl alcohol and these contents added to the collected feces. All remaining tissues were labeled carcass. Saturated potassium hydroxide solution (1 cc per g tissue) was added to each tissue sample and the fats were saponified. After saponification, the solutions were made acid to methyl red with sulfuric acid and the fatty acids extracted with ether. Aliquots of the ether extracts were mounted on lens-paper-covered aluminum discs for determination of their radioactivity, as described elsewhere.[7]

The time required to yield 1024 counts was determined several times for each sample. A Geiger-Müller tube with a mica window of 1.7 mg per sq. cm was used. All counting rates were at least several times that of background.

Results. The rapidity with which the intravenously injected tripalmitin is oxidized by fasted rats is shown in Fig. 1. Significant amounts of C^{14} appeared in the expired CO_2 as early as one hour, and by the time 24 hours had elapsed, from 36 to 59% of the sixth carbon atoms of the palmitic acid molecules were recovered as $C^{14}O_2$. The rate of elimination of $C^{14}O_2$ was not uniform throughout the period studied. It was most rapid between the second and fourth hours—from 19 to 25% of the total 24-hour elimination was expired during these 2 hours.

The recovery, at the end of 24 hours, of the injected C^{14} among the fatty acids isolated from various tissues is shown in Table I. Significant amounts, namely, 6-8%, were found in the areas designated as fatty depots and of these, the largest amount was present in the subcutaneous fat. If these values are considered in conjunction with the amount of C^{14} undoubtedly present in intermuscular fat of the carcass, there can be no doubt that fatty depots constitute a most important metabolic pathway for *intravenously* administered fat.

The only other tissue that contained appreciable amounts of the injected C^{14} was the liver. One day after the administration of the labeled palmitic acid, approximately 3-6% of the C^{14} was recovered in the fatty acid fraction of this tissue. Negligible amounts of C^{14} were found in the brain, spleen, lungs, and kidneys.

That the intravenously administered emulsified tripalmitin is available for phospholipid formation is shown in Table II. In rats 3 and 4, the major portion (70-80%) of the C^{14}-labeled fatty acids in the liver and small and large intestine had been incorporated into phospholipids. This was also true for the small intestine of rats 1 and 2, but in the case of

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[7] Entenman, C., Lernér, S. R., Chaikoff, I. L., and Dauben, W. G., Proc. Soc. Exp. Biol. and Med., in press.

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[8] Geyer, R. P., Chipman, J., and Stare, F. J., *J. Biol. Chem.*, 1948, **176**, 1469.

16935

A Fat Emulsion for Intravenous Feeding.*

S. R. Lerner, I. L. Chaikoff, and C. Entenman.

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Attempts to utilize fat for intravenous feeding, because of its high caloric content, have led to the development of many types of fat emulsions.[1-9] The special problems encountered in such usage involve the dispersion of the fat into suitable particle sizes (0.5 to 1 μ) and the stabilization of that dispersion. The former has been achieved by high pressure homogenization of such fats as coconut, olive, corn, and butter oils. Egg or soya phosphatides have, in most cases, been used as stabilizers.

McKibbin *et al.* devised an emulsion of coconut oil which was stabilized with soya phosphatides.[6] It was well tolerated when injected slowly, but foreign body responses were observed following chronic injections. The investigators reported, however, that impurities in the soya phosphatides caused the foreign body reactions and that simple purification freed the emulsion of toxicity.[10] These findings have been confirmed by Ng in this laboratory.[11] He found that daily intravenous injections of a commercially prepared soya phosphatide, for 15-25 days, produced a mild hemolysis and a lipid-storage reaction in the reticulo-endothelial system. Use of the fraction of this commercial preparation which was soluble in 95% ethyl alcohol at 60° did not prevent the untoward reactions.

Meng and Freeman devised an emulsion of butter oil stabilized by the presence of a polyalcohol derivative and soya phosphatides, in an oil phase, and sodium cholate in an aqueous phase.[7] This emulsion was tolerated during its intravenous administration and, as shown by postmortem examination, resulted in only minimal changes in lungs and kidneys of dogs that had received daily injections for several weeks.

Shafiroff and Frank prepared an emulsion of coconut oil in a menstruum composed of 6% gelatin, 5% protein hydrolysate, and 5% glucose.[8,9] Apparently it was well tolerated for they observed no evidence of toxicity.

In the present investigation, a fat emulsion not hitherto used for intravenous feeding is described. This consisted of an olive oil dispersion stabilized by means of glycerol monostearate. The daily intravenous injection of this emulsion for as long as 2 to 4.5 weeks produced only minimal reactions in dogs as judged by microscopic examination of their tissues.

Experimental. Preparation of emulsion. Two parts of olive oil and one part glycerol monostearate† were heated, with stirring, until

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* Aided by a grant from the Cutter Laboratories, Berkeley.

[1] Nomura, T., *Tohoku J. Exp. Med.*, 1929, **12**, 247.

[2] Holt, L. E., Jr., Tidwell, H. C., and Scott, T. F., *J. Pediatrics*, 1935, **6**, 151.

[3] Clark, D. E., and Brunschwig, A., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 329.

[4] Myers, R. J., and Blumberg, H., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 79.

[5] Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, **48**, 395.

[6] McKibbin, J. M., Pope, A., Thayer, S., Ferry, R. M., Jr., and Stare, F. J., *J. Lab. Clin. Med.*, 1945, **30**, 488.

[7] Meng, H. C., and Freeman, S., *J. Lab. Clin. Med.*, 1948, **33**, 689.

[8] Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.

[9] Shafiroff, B. G. P., Baron, H., and Roth, E., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 387.

[10] Geyer, R. D., Mann, G. V., Young, J., Kinney, T. D., and Stare, F. J., *L. Lab. Clin. Med.*, 1948, **33**, 163.

[11] Ng, E., unpublished observations.

† A commercial preparation manufactured by Glyco Products Co., Inc., Brooklyn, New York, and designated Aldo 28, was used. This product is glycerol monostearate modified by the inclusion of a small amount of a sodium soap. Emulsions prepared with unmodified glycerol monostearate gelled a few hours after autoclaving.

a clear solution was obtained. This melt was then added to a sufficient volume of 5% glucose to yield a final concentration of 10% fat, and the mixture was stirred in a Waring blender for 3-5 minutes. Three gallons of this preliminary emulsion were passed through a dairy homogenizer 5 times at 2500 lb. per sq. inch. The diameters of the fat particles of the final emulsion were determined by visual measurement with an ocular micrometer and, as a rule, found to be one micron or less. Particles as large as 2 μ were only occasionally observed. The pH of this emulsion was 7.0. Such emulsions contained about 10% fatty acids of which 2/3 were derived from olive oil and 1/3 from glycerol monostearate. Bottling in cotton-stoppered 500 cc Erlenmeyer flasks, autoclaving for 30 minutes at 5 lb., and cooling in an ice bath produced a sterile emulsion that creamed only slightly after 2 months. The emulsion was stored at room temperature.

Treatment of dogs. For several days before injection and throughout the injection period, the dogs were fed once daily a mixture composed of 40 g of lean horse meat per kg body weight, 20 g of sucrose, 20 g of bone ash, one cc of fish oil,‡ and 10 g of yeast. Before the start of the injection, samples of blood were withdrawn for determination of red cell counts and hemoglobin concentration. Liver function also was measured by means of the Rose Bengal test as described by Hough and Freeman.[12]

The dogs were injected with 200 cc of the fat emulsion, either daily or 6 days per week. The dogs received, in all, 16 to 31 intravenous injections. A few days before the last injection, evidence of toxicity was sought by determination of red cell counts, hemoglobin concentrations of blood, and of liver function.

Methods. The method used for the determination of fatty acid contents of plasma and

TABLE 1.
Effect of Intravenous Injections of a Fat Emulsion on Dogs.
(Each dog received 200 cc of 10% fat emulsion at each injection.)

		Wt		Hemoglobin		Red blood cell count		Rose Bengal clearance		
Dog	No. of inj.	Before inj. started, kg	At end of inj. period, kg	Before inj. started, g per 100 ml	On indicated day of inj. period, g per 100 ml	Before inj. started, $\times 10^6$	On indicated day of inj. period, $\times 10^6$	Before inj. started	On indicated day of inj. period	Liver fatty acid, % wet wt
1	13	12.7	12.5	16.0	13.0 (13)*	8.1	7.1 (13)*	130†	114 (13)*	3.1
2	24	8.7	8.5	13.2	10.8 (20)	7.1	4.7 (20)	130	82 (20)	2.9
3	16	15.1	15.4	15.6	12.5 (15)	7.6	6.6 (15)	114	110 (15)	3.2
4	16	10.2	10.3	14.0	12.6 (15)	7.7	6.4 (15)	114	82 (15)	4.1
5	31	13.2	12.5	16.0	9.5 (31)	8.7	5.2 (31)	104	108 (31)	—

* Numbers in parentheses indicate the day of the injection when test made.
† Values lower than 100 indicate impaired excretion.[12]

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‡ Each cubic centimeter of the fish oil (sardilene) contained not less than 100 A.O.A.C. chick units of vitamin D and 600 U.S.P. units of vitamin A.

[12] Hough, V. H., and Freeman, S., *Am. J. Physiol.*, 1942, 138, 184.

liver has been recorded elsewhere.[13,17] Red cell counts and hemoglobin concentrations of blood were determined as described by Gradwohl.[14]

Results. The 200 cc of the emulsion which each dog received daily by intravenous drip contained 13.3 g of olive oil and 6.7 g of glycerol monostearate, or a total of 20 g of fatty acids. It was administered at a rate of about 1 cc per minute per kg of body weight. No evidence of distress other than a slight restlessness was observed during the injections which occupied, as a rule, from 20 to 30 minutes. Immediately after completion of the injection, the dogs were fed their daily ration which was readily consumed.

Five dogs were used in this investigation (Table I). They received 16 to 31 daily injections. Red cell counts, hemoglobin concentrations of the blood, and liver function were measured on the day before the injections were begun and at designated days near the end of the injection period.

Before the injections were begun, the hemoglobin content of the blood of the 5 dogs ranged from 13.2 to 16.0 g per 100 cc. These values are well within the range reported by other investigators.[6,7,15] The concentration of hemoglobin in all 5 dogs had decreased by the time the injections had continued for 13 to 31 days. This is in keeping with the observed fall of the red cell counts. Similar hematological findings were reported by McKibbin *et al.*[6] and by Meng and Freeman[7] in their dogs that received injections of fat emulsions. Despite the slight anemia a weight loss was not observed in 4 dogs that received as many as 31 daily infusions. Dogs 1, 3, and 5 showed no evidence of impaired liver function as judged by the rate at which injected Rose Bengal was lost from the blood stream.

TABLE II.
Rate of Disappearance of Injected Fat from the Blood Stream of the Dog.
(All lipid values expressed as mg per 100 cc plasma).

Dog 1*			Dog 2†			Dog 3†		Dog 4†		Dog 5‡			
Interval after inj., min.	Total fatty acids	Total cholesterol	Interval after inj., min.	Total fatty acids	Total cholesterol	Interval after inj., min.	Total fatty acids	Interval after inj., min.	Total fatty acids	Interval after inj., min.	Total fatty acids	Total cholesterol	Phospholipid
—12§	356	166	—6§	338	167	—1§	317	—2§	194	—21§	456	213	364
9	1510	169	2	1310	134	5	1170	4	1940	9	1110	163	282
94	665	172	38	819	159	44	710	23	2000	49	766	198	321
246	412	167	72	582	142	99	410	69	1150	136	526	199	328
535	405	172	132	465	155	160	317	152	402	202	431	196	328
			189	472	157			217	293				

* This dog received a total of 13 injections and the data shown above are for the eighth injection.
† The measurements recorded above were made on the day of the last injection.
‡ This dog received a total of 31 injections and the data shown above are for the 26th injection.
§ Minus values indicate the time in minutes before the injections were started.

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[13] Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1937, **119**, 423.

[14] Gradwohl, R. B. H., Clinical Laboratory Methods and Diagnosis, 3rd ed., 1943.

[15] Allison, J. B., *Trans. N. Y. Acad. Sci.*, Ser. II, 1946, **8**, 260.

[16] Entenman, C., Chaikoff, I. L., and Zilversmit, D. B., *J. Biol. Chem.*, 1946, **166**, 15.

[17] Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **106**, 267.

The excretion of the dye by dogs 2 and 4 was 82% of normal.

The rate of disappearance of the injected fat from the blood stream was measured for each dog and the results are shown in Table II. The first examination of plasma fatty acids was made from 2 to 9 minutes after the completion of the injection, and at these times fatty acid concentrations were between 1110 and 1940 mg %. Utilization of the injected fat, as judged by its disappearance from the blood stream, was rapid in all 5 dogs, and by the time 4 hours had elapsed, the concentration of plasma fatty acids approached the preinjection levels.

The dogs were sacrificed by an overdose of nembutal 24 hours after the last injection. All organs were examined grossly and lungs, liver, kidney, spleen, small intestine, and heart studied microscopically. The lungs of all 5 dogs were free of lipoid granulomatous lesions and the lungs of only 2 dogs showed a few inflammatory foci. The reticulo-endothelial elements of the spleens and livers contained moderate amounts of blood pigments, a finding that indicates that some hemolysis had occurred. In dog 4, lipid-like material was found in the reticulo-endothelial cells of the liver and in the capillary loops of occasional glomerular tufts. Dog 2 had a few shrunken and scarred glomeruli adherent to the capsule. Protein containing fluid was found in the glomerular and tubular spaces. Dog 5 was pregnant and 2 of its 6 fetuses were dead. The fatty acid contents of the livers of dogs 1-4 were normal (Table I). The liver of dog 5, as judged by microscopic examination, was free of abnormal amounts of fat.

Comment. It is shown here that by the use of glycerol monostearate as stabilizer, an olive oil emulsion in which the fat remains dispersed for several months can be easily prepared. This emulsion is well tolerated by dogs when injected intravenously at rates of 1 cc per minute per kg, and is rapidly removed from the blood stream. Although dogs that had received as many as 31 injections were not free of tissue responses, the reactions observed were quite mild in character.

In confirmation of earlier findings,[1,2,4,7] it was observed here that intravenously administered fat rapidly disappears from the blood stream. The rate at which this occurs suggests that the administered fat is taken up by the liver for it has been shown that lipids disappear slowly, if at all, from the plasma of hepatectomized dogs.[16]

Summary. 1. The preparation of a stable olive oil emulsion in which glycerol monostearate was employed as the emulsifying agent is described. 2. Observations on its use for intravenous feeding in dogs are recorded.

16936

Excretion of 17-Ketosteroids by Patients Suffering from Poliomyelitis.*

SAUL L. COHEN.

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It has long been recognized that the incidence of poliomyelitis is greater for children than for adults (Fanconi[1]). Since the excretion of 17-ketosteroids increases rapidly during adolescence it was thought possible that a low androgen excretion may be directly or indirectly associated with an increased incidence to poliomyelitis. The excretion of 17-ketosteroids by poliomyelitis patients was, therefore, investigated.

The urine specimens from a total of 39

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* Aided by a grant from the National Foundation for Infantile Paralysis.

[1] Fanconi, G., *Die Poliomyelitis und Ihre Grenzgebiete*, Benno Schwabe and Co., Basel, Switzerland.

TABLE I.
Excretion of 17-Ketosteroids by Patients Suffering from Poliomyelitis.

No. of patients	Sex	Age range	Type of poliomyelitis	Mg 17-Ks. excreted/24 hr range	avg
10	Male	5-13	Bulbar (4)	0.2-1.8	1.0
			Spinal (4)	0.8-4.8	2.5
			Bulbar-Spinal (2)	0.9-2.0	1.5
1	Female	7	Spinal	2.3	
15	Male	14-39	Bulbar (3)	11.4-36.0	19.0
			Spinal (8)	3.4-27.1	10.3
			Bulbar-Spinal (4)	14.9-22.1	19.0
13	Female	14-35	Bulbar (3)	3.1-7.2	5.4
			Spinal (8)	2.0-21.1	8.0
			Bulbar-Spinal (2)	8.6-14.6	11.6

patients entered at the University of Minnesota Hospital during the 1946 epidemic were studied. Of these, 11 were under age 13, 11 were females over age 14, and 15 were males over age 14. In some cases urine specimens were collected from the same patient on more than one occasion and these were subjected to separate analyses. All specimens had been stored in a frozen condition since the time of collection. The 17-ketosteroid fractions were prepared from 200-400 cc aliquots of 24-hour specimens by a modified Callow technic (Callow, *et al.*[2]) and were colorimetrically assayed by the technique of Holtorff and Koch[3]. The results are shown in Table I.

The values for the 17-ketosteroid excretion of the patients suffering from poliomyelitis for the most part fall within the normal ranges reported by others (cf. Talbot and Butler[4]) and as found in our own laboratory. These data thus tend to indicate that there is no relationship between the condition of poliomyelitis and the excretion levels of 17-ketosteroids. No significant differences were observed for the 17-ketosteroid excretion of patients suffering from different types of poliomyelitis.

Summary. The 17-ketosteroids excreted into the urine by patients suffering from different types of poliomyelitis showed no significant variations from normal excretion levels.

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[2] Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, **32**, 1312.

[3] Holtorff, A. F., and Koch, F. C., *J. Biol. Chem.*, 1940, **135**, 377.

[4] Talbot, H. B., and Butler, A. M., *J. Clin. Endocrinology*, 1942, **2**, 724.

16937

Growth Promoting Activity of Vitamin B_{12} in Rats Receiving Thyroid Substance.

GLADYS A. EMERSON. (With the technical assistance of Marilyn A. Bingemann.)
From the Merck Institute for Therapeutic Research, Rahway, N. J.

The retarded growth observed in young rats fed toxic doses of thyroid powder is counteracted by liver.[1,2] Extracted Liver Residues (Wilson) had "anti-thyrotoxic" activity when fed at a level of 10% in a diet containing 22% casein.[3] Reticulogen also was effective in reversing the growth retarding action of thyroid substance in chicks fed a diet devoid of animal protein.[4,5]

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[1] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

[2] Betheil, J. S., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

[3] Ershoff, B. H., *Arch. Biochem.*, 1947, **15**, 365.

TABLE I.
Effect of Vitamin B_{12} on Growth of Male Rats Receiving Thyroid Powder.

Groups of 10 male rats each	Supplement	Gain in wt 15 days, g	Avg daily food intake 15 days, g
H_4 (Casein)	None	65	16
282 (Soybean meal)	"	49	17
286 (Soybean meal + 0.25% thyroid powder)	"	28	17
" " " " " "	5.8 mg cobalt nitrate	27	—
" " " " " "	0.5 γ B_{12} p.o.*	79	17
" " " " " "	" " " s.c.†	74	—
" " " " " "	0.25 " " p.o.	72	—
" " " " " "	" " " s.c.	73	—
" " " " " "	0.125 " " p.o.	65	—
" " " " " "	" " " s.c.	71	—
" " " " " "	0.0625 " " p.o.	51	—
" " " " " "	" " " s.c.	50	—

* *Per os.*
† Subcutaneously.

Vitamin B_{12}, a crystalline compound which has been isolated from both liver[6] and fermentation sources,[7] neutralizes the growth inhibiting effect observed in immature rats following the feeding of a diet containing soybean meal and thyroid powder. The basal diet (282) employed in these studies had the following composition: soybean meal, 60; salts, 4; dextrose, 24; crisco, 10; cod liver oil, 2 g/100 g and micronutrients: thiamine, 1; riboflavin, 2; pyridoxine, 1; Ca pantothenate, 10; nicotinamide, 10; inositol, 5; p-aminobenzoic acid, 30; choline, 100; biotin, 0.05; pteroylglutamic acid, 0.20; α-tocopherol, 14.2; 2 Me 1:4 naphthoquinone, 14.2 mg/100 g. Desiccated thyroid U.S.P. (Armour) replaced dextrose at a level of 0.25% in diet 286. A purified ration (H_4),* containing 24% Labco casein, served as a standard for comparison.

Male rats, averaging 40 g in weight, were placed at weaning on each of the 3 dietary regimens. After 28 days on experiment the weight increments in g for the several groups were as follows: Diet H_4 (24% casein), 130; diet 282 (60% soybean meal), 120; and diet 286 (60% soybean meal and 0.25% thyroid powder), 80. Following this depletion period the rats receiving diet 286 were segregated into groups as indicated in Table I.

The animals receiving 0.125 γ vitamin B_{12} daily in addition to diet 286 grew over a 15-day test period at a rate which was more than twice that of the controls. Their weight gain, over this period, approximated that of animals receiving the diet containing casein and exceeded that made by the rats fed the ration containing soybean meal without thyroid substance. This level appeared to approach the optimal as indicated by the fact that the feeding of higher levels of B_{12} resulted in only slightly increased weight increments. Vitamin B_{12} appeared to be utilized equally well when administered by either the oral or subcutaneous routes. Rats given cobalt nitrate by the oral route made

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[4] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 400.

[5] Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 419.

[6] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

[7] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **108**, 634.

* Composition of Diet H_4	g/100 g
Casein (Labco)	24
Dextrose	60
Salts (U.S.P. No. 1)	4
Hydrogenated veg. oil	10
Cod liver oil	2

Micronutrients, mg/100 g: thiamine, 1; riboflavin, 2; pyridoxine, 1; Ca pantothenate, 10; nicotinamide, 10; inositol, 5, and choline chloride, 100.

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Summary. The 17-ketosteroids excreted into the urine by patients suffering from different types of poliomyelitis showed no significant variations from normal excretion levels.

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16937

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,, ,, ,, ,, ,, ,,	0.5 γ B_{12} p.o.*	79	17
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.†	74	—
,, ,, ,, ,, ,, ,,	0.25 ,, ,, p.o.	72	—
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.	73	—
,, ,, ,, ,, ,, ,,	0.125 ,, ,, p.o.	65	—
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.	71	—
,, ,, ,, ,, ,, ,,	0.0625 ,, ,, p.o.	51	—
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.	50	—

* *Per os.*
† Subcutaneously.

Vitamin B_{12}, a crystalline compound which has been isolated from both liver[6] and fermentation sources,[7] neutralizes the growth inhibiting effect observed in immature rats following the feeding of a diet containing soybean meal and thyroid powder. The basal diet (282) employed in these studies had the following composition: soybean meal, 60; salts, 4; dextrose, 24; crisco, 10; cod liver oil, 2 g/100 g and micronutrients: thiamine, 1; riboflavin, 2; pyridoxine, 1; Ca pantothenate, 10; nicotinamide, 10; inositol, 5; p-aminobenzoic acid, 30; choline, 100; biotin, 0.05; pteroylglutamic acid, 0.20; α-tocopherol, 14.2; 2 Me 1:4 naphthoquinone, 14.2 mg/100 g. Desiccated thyroid U.S.P. (Armour) replaced dextrose at a level of 0.25% in diet 286. A purified ration (H_4),* containing 24% Labco casein, served as a standard for comparison.

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The animals receiving 0.125 γ vitamin B_{12} daily in addition to diet 286 grew over a 15-day test period at a rate which was more than twice that of the controls. Their weight gain, over this period, approximated that of animals receiving the diet containing casein and exceeded that made by the rats fed the ration containing soybean meal without thyroid substance. This level appeared to approach the optimal as indicated by the fact that the feeding of higher levels of B_{12} resulted in only slightly increased weight increments. Vitamin B_{12} appeared to be utilized equally well when administered by either the oral or subcutaneous routes. Rats given cobalt nitrate by the oral route made

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[5] Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 419.

[6] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

[7] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **108**, 634.

* Composition of Diet H_4	g/100 g
Casein (Labco)	24
Dextrose	60
Salts (U.S.P. No. 1)	4
Hydrogenated veg. oil	10
Cod liver oil	2

Micronutrients, mg/100 g: thiamine, 1; riboflavin, 2; pyridoxine, 1; Ca pantothenate, 10; nicotinamide, 10; inositol, 5, and choline chloride, 100.

weight gains comparable with those of the controls indicating that cobalt could not be employed under these conditions in the biosynthesis of vitamin B_{12}.

It is of interest to note that the food consumption over the 15-day test period for the rats receiving 0.5 γ of vitamin B_{12} daily and those fed the unsupplemented diet but without thyroid powder were the same. The red and white blood cell counts in both the control group and in the groups receiving vitamin B_{12} fell within the normal limits.

Summary. Vitamin B_{12} counteracts the growth retarding effect of thyroid powder when fed in conjunction with a diet devoid of animal protein. The vitamin is equally effective when administered by either the oral or the subcutaneous route.

16938

Entrance of Intravenously Injected Sucrose into the Cerebrospinal Fluid.*

ROGER S. HUBBARD AND JOHN ZOLL.

From the Department of Pharmacology, the Medical School, University of Buffalo, and the Millard Fillmore Hospital, Buffalo, N. Y.

Sucrose injected intravenously into human subjects can be recovered almost quantitatively from the urine.[1] It has been used as an osmotic diuretic drug[2] although its toxic actions greatly limit its usefulness.[3] The excretion of the sugar by the kidneys appears to result from quantitative filtration through the glomeruli, and, since the compound is very little affected by tubular reabsorption, its rate of excretion has been used to determine glomerular filtration[4] although sucrose is not apparently as satisfactory as inulin for this purpose.

Besides its action as a diuretic, it has been used in the study of body fluids. It appears not to penetrate cells readily[1] and to be contained almost solely in the extra-cellular fluid. Lavietes and his coworkers[5] have suggested that it be used to determine total extracellular fluid when large accumulations of abnormal fluid are absent. On the other hand sucrose appears to penetrate poorly into the spinal canal[6] and has been injected intravenously to reduce intracranial pressure by osmotic action.[7]

Hubbard and Anderson[8] developed a specific method for determining sucrose, injected large amounts of the sugar into human subjects who showed unusual accumulations of extra-cellular fluid, and compared the concentrations of sucrose in blood plasma and extracellular fluid samples withdrawn after various time intervals had elapsed. They found that sucrose diffused readily into extracellular

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* Our thanks are due to Dr. William Beswick for the opportunity for studying patients receiving treatment for increased intracranial pressure and to Dr. Wilbert H. Spencer for assistance with the bacteriological part of the study.

[1] Keith, N. M., and Powers, M. H., *Am. J. Physiol.*, 1937, **120**, 203.

[2] Goodman, L., and Gilman, A., The Pharmacologic Basis of Therapeutics, New York, 1941, p. 632.

[3] Helmholtz, H. F., *J. Pediat.*, 1933, **3**, 144; Anderson, W. A. D., and Bethea, W. R., *J.A.M.A.*, 1940, **114**, 983; Lindberg, H. S., Wald, M. H., and Barker, M. H., *Arch. Int. Med.*, 1939, **63**, 907.

[4] Joliffe, N., Shannon, J. A., and Smith, H. W., *Am. J. Physiol.*, 1932, **100**, 301; Shannon, J. A., and Smith, H. W., *J. Clin. Invest.*, 1935, **14**, 393.

[5] Lavietes, P. H., Bourdillon, J., and Klinghoffer, K. A., *J. Clin. Invest.*, 1936, **15**, 261.

[6] Gregerson, M. I., and Wright, L., *Am. J. Physiol.*, 1935, **112**, 97.

[7] Bulloch, M. T., Gregerson, M. I., and Kiney, R., *Am. J. Physiol.*, 1935, **112**, 82; Masserman, J. H., *Bull. Johns Hopkins Hosp.*, 1935, **57**, 12; Jackson, H., Dickerson, D., and Gunther, A., *Am. Surg.*, 1937, **106**, 161.

[8] Hubbard, R. S., and Anderson, R. K., *Am. J. Physiol.*, 1942, **137**, 722.

TABLE I.
Comparison of Sucrose in Serum and Spinal Fluid.

No.	Pathological condition	Time after sucrose inj., min.	Sucrose in serum, mg per 100 cc	Sucrose in spinal fluid, mg per 100 cc
1.	Brain tumor post operative	15	134	trace*
2.	Chronic subdural hematoma	25	316	"
3.	Polyneuritis	45	212	"
4.	Subarachnoid hemorrhage. Ruptured aneurysm	60	264	4.0
5.	Cerebral thrombosis and subarachnoid hemorrhage	90	170	trace*
6.	Cerebral thrombosis	90	154	2.5
7.	Head injury with cerebral laceration	90	50	3.0
8.	Subarachnoid hemorrhage. Ruptured aneurysm	120	54	10.8
9.	Brain tumor post operative	180	22.3	trace*
10.	Encephalitis	180	30	1.0

* Approximately 0.5 mg per 100 cc.

fluid, but that equilibrium was reached slowly. The extracellular fluid formed a reservoir out of which sucrose diffused very slowly, and in one instance its presence was demonstrated in fluid and plasma 97 hours after the material was injected, while in normal subjects negative results were obtained in a much shorter time. In the 7 patients studied 20 hours or more after the sugar was given the concentration of the sugar was higher in the abnormal fluid than in the plasma.

It seemed desirable to extend the study to the equilibrium between blood and spinal fluid, for (A) a previous experiment[9] had demonstrated the presence of sucrose in the spinal fluid of a child who died shortly after an injection of the sugar, and (B) a late rise in intracranial pressure, following the initial drop, had been observed in some patients to whom sucrose had been given intravenously. In spite of the low values for spinal fluid sucrose reported by others, it seemed possible that the spinal fluid might serve as a reservoir for the sugar since such an effect had been observed in edema, peritoneal, and pleural fluids.

The method used was similar to that employed by Hubbard and Anderson.[8] 25 g of sucrose, given as 50 cc of a 50% solution were injected intravenously into 10 patients suffering from various clinical conditions. Specimens of blood and fluid were withdrawn after various periods and analyzed for sucrose. In each instance duplicate specimens were fermented, one by a strain of *Escherichia coli* which fermented all common sugars, and the other by a strain which did not destroy sucrose, but did ferment all other sugars present in body fluids. After clarification by Somogyi's zinc method[10] sucrose was determined by the resorcinol method of Roe.[11] The method gave satisfactory recovery of sucrose added to plasma and to spinal fluid, and appeared to demonstrate successfully concentrations greater than about 0.5 mg per 100 cc.

The results are given in Table I. It is evident that sucrose was present in spinal fluid in very small amounts, and that only when a hemorrhage was present could the amount found be considered significant. Even then the concentrations were so low that the authors do not believe they could explain any secondary rise in intracranial pressure, even if they had persisted until all the sucrose had been excreted from the blood by the kidneys. The figures are probably influenced by a slow diffusion of sucrose through the spinal fluid after it had entered the canal, but it seems improbable that this factor is adequate to explain the great difference between these figures and the values reported by Hubbard and Anderson for other extracellular fluids. It seems certain that, in contrast with the ease with which sucrose passes out of the capillaries into accumulations of body fluid and is filtered through glomeruli, it normally passes weight gains comparable with those of the controls indicating that cobalt could not be employed under these conditions in the biosynthesis of vitamin B_{12}.

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[9] Hubbard, R. S., and Terplan, K., *Arch. Path.*, 1935, **20**, 307.

[10] Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

[11] Roe, J. H., *J. Biol. Chem.*, 1934, **107**, 15.

It is of interest to note that the food consumption over the 15-day test period for the rats receiving 0.5 γ of vitamin B_{12} daily and those fed the unsupplemented diet but without thyroid powder were the same. The red and white blood cell counts in both the control group and in the groups receiving vitamin B_{12} fell within the normal limits.

Summary. Vitamin B_{12} counteracts the growth retarding effect of thyroid powder when fed in conjunction with a diet devoid of animal protein. The vitamin is equally effective when administered by either the oral or the subcutaneous route.

16938

Entrance of Intravenously Injected Sucrose into the Cerebrospinal Fluid.*

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Sucrose injected intravenously into human subjects can be recovered almost quantitatively from the urine.[1] It has been used as an osmotic diuretic drug[2] although its toxic actions greatly limit its usefulness.[3] The excretion of the sugar by the kidneys appears to result from quantitative filtration through the glomeruli, and, since the compound is very little affected by tubular reabsorption, its rate of excretion has been used to determine glomerular filtration[4] although sucrose is not apparently as satisfactory as inulin for this purpose.

Besides its action as a diuretic, it has been used in the study of body fluids. It appears not to penetrate cells readily[1] and to be contained almost solely in the extra-cellular fluid. Lavietes and his coworkers[5] have suggested that it be used to determine total extracellular fluid when large accumulations of abnormal fluid are absent. On the other hand sucrose appears to penetrate poorly into the spinal canal[6] and has been injected intravenously to reduce intracranial pressure by osmotic action.[7]

Hubbard and Anderson[8] developed a specific method for determining sucrose, injected large amounts of the sugar into human subjects who showed unusual accumulations of extra-cellular fluid, and compared the concentrations of sucrose in blood plasma and extracellular fluid samples withdrawn after various time intervals had elapsed. They found that sucrose diffused readily into extracellular

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* Our thanks are due to Dr. William Beswick for the opportunity for studying patients receiving treatment for increased intracranial pressure and to Dr. Wilbert H. Spencer for assistance with the bacteriological part of the study.

[1] Keith, N. M., and Powers, M. H., *Am. J. Physiol.*, 1937, **120**, 203.

[2] Goodman, L., and Gilman, A., The Pharmacologic Basis of Therapeutics, New York, 1941, p. 632.

[3] Helmholtz, H. F., *J. Pediat.*, 1933, **3**, 144; Anderson, W. A. D., and Bethea, W. R., *J.A.M.A.*, 1940, **114**, 983; Lindberg, H. S., Wald, M. H., and Barker, M. H., *Arch. Int. Med.*, 1939, **63**, 907.

[4] Joliffe, N., Shannon, J. A., and Smith, H. W., *Am. J. Physiol.*, 1932, **100**, 301; Shannon, J. A., and Smith, H. W., *J. Clin. Invest.*, 1935, **14**, 393.

[5] Lavietes, P. H., Bourdillon, J., and Klinghoffer, K. A., *J. Clin. Invest.*, 1936, **15**, 261.

[6] Gregerson, M. I., and Wright, L., *Am. J. Physiol.*, 1935, **112**, 97.

[7] Bulloch, M. T., Gregerson, M. I., and Kiney, R., *Am. J. Physiol.*, 1935, **112**, 82; Masserman, J. H., *Bull. Johns Hopkins Hosp.*, 1935, **57**, 12; Jackson, H., Dickerson, D., and Gunther, A., *Am. Surg.*, 1937, **106**, 161.

[8] Hubbard, R. S., and Anderson, R. K., *Am. J. Physiol.*, 1942, **137**, 722.

TABLE I.
Effect of Different Amounts of A-Methopterin on the Growth of the Sarcoma 180.

Dose in mg/kg/day	No. of groups of 5 mice	No. of mice	Deaths during inj. period		Effect on tumor*			
			No.	%	Inconclusive	Marked inhibition	Moderate effect	No effect
2.5	6	30	14	46.6	2*	4	0	0
2.0	15	75	14	18.6	2	9	4	0
1.5	31	155	11	7.1	0	16	11	4
1.0	8	40	2	5.0	0	1	4	3

* The tumor effects are expressed as the result obtained in each group of 5 mice. Thus, the numeral 2 indicates two groups of 5 mice each.

the volume of such a tumor would be 1/64 or less of the control if tumors may be considered spherical.

The animals were again weighed and their tumors measured at the end of the second week.

Results. Groups of 5 mice implanted with the Sarcoma 180 one day previously were injected intraperitoneally with daily doses of A-Methopterin at the following levels: 2.5 mg, 2.0 mg, 1.5 mg, and 1 mg/kg. Deaths due to the toxicity of the material were recorded during the 7-day period of injection. Tumor measurements were made at the end of the period and the effect of the substance on the tumor was evaluated. These data are summarized in Table I.

It is clear that A-Methopterin has a definite inhibitory effect on the growth of Sarcoma 180 at all the levels of drug above 1 mg/kg/day. The drug killed 46% and 18%, respectively, in the groups of mice receiving 2.5 and 2.0 mg/kg/day. At the lower dosages 7% and 5% of the mice receiving 1.5 and 1.0 mg died during the treatment. Male mice appeared to tolerate the drug better than female mice. The best results were obtained at the 1.5 mg dose where a definite effect was noted in almost all instances with little evidence of toxicity. The average weight loss of this group was 1.0 g as contrasted to a gain of 0.5 g for the controls. Fig. 1 shows the typical inhibition effect on the tumor of the injection of A-Methopterin at the level of 1.5 mg/kg/day.

The tumors have never been completely destroyed. When the injections are stopped, the small tumors grow normally.

When the tumors were allowed to grow for 3 to 7 days before the injections were begun, inhibition of growth was apparent in practically all instances. Of the 15 groups of mice receiving 2 mg/kg/day, inhibition was marked in 3 groups, moderate in 11 groups and absent in one group. Of the 24 groups receiving 1.5 mg/kg/day, inhibition was marked in 7, moderate in 16, and absent in one. The death rates for the 2 dosages were 12% and 16%, respectively. It is doubtful that these figures represent true toxicity deaths, because many of the tumors were so large at the time injections were initiated, that some of the mice probably died from the effects of tumor growth. The effect of A-Methopterin was definite although not as marked as in the groups of mice injected 24 hours after implantation.

In a few experiments a single injection of 50 mg/kg was administered when the tumor had grown one day. An inhibitory effect was evident when the tumors were measured a week later. In one group implanted 24 hours previously which received one injection of 50 mg/kg, small doses (0.5 mg/kg/day) were administered for 7 days. When the tumors were measured, they were very small but grew when the injections were stopped. The development of 7-day-old tumors was not affected by the injection of the single large dose.

Discussion. A-Methopterin has been shown to have a definite and consistent inhibitory effect on the growth of the transplantable mouse Sarcoma 180. In another report (Sugiura, Moore, Stock)[4] the folic acid antagonist, Aminopterin, was shown to have a from the blood into the spinal fluid in very small amounts.

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[4] Sugiura, K., Moore, A., and Stock, C. C., *Cancer*, in press.

Summary. After intravenous injection sucrose occurs in the spinal fluid in very low concentration. Even when hemorrhages into the central nervous system occur, the concentration does not approach that found in blood.

16939

Inhibition of Development of Sarcoma 180 by 4-Amino-N^{10}-Methyl Pteroylglutamic Acid.*

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In view of the extensive developments among the anti-metabolites it was a natural consequence that cancer chemotherapy studies should be initiated with analogs of folic acid. Interest in the folic acid antagonists has been stimulated by reports of the effectiveness of Aminopterin in the treatment of leukemia (Farber).[1] In an intensive screening program during the past year a large number of analogs of folic acid and various related simple compounds have been studied against mouse leukemia[2] and various mouse and rat tumors with particular emphasis on the Crocker mouse Sarcoma *180*. Thus far, one folic acid antagonist,[3] 4-Amino-N^{10}-methyl pteroylglutamic acid (A-Methopterin),† has been outstanding in its ability to inhibit the growth of Sarcoma 180. The preliminary results of the study are presented.

Materials and methods. The material was tested for ability to inhibit the growth of the Sarcoma 180 and also for its ability to damage a well established tumor.

Carefully selected tumors were cut into 2 mm cubes and implanted subcutaneously into the axillary region of groups of 5 weighed and marked white mice. In the inhibition tests, intraperitoneal injections of 0.5 cc of different doses of A-Methopterin were begun 24 hours later. In the therapeutic test, injections were begun after 3 to 7 days of tumor growth. In all instances, injections were given twice daily at 6-hour intervals for 7 consecutive days. If the animals showed definite signs of a toxic effect of the drug, administration was omitted until recovery had taken place. At the higher dosage (2.5-2.0 mg/kg/day) 3 or 4 omissions were often necessary. Below this level the drug was well tolerated.

Following the period of injections the animals were weighed and the size of their tumors determined by measurement in 2 diameters with calipers. The size of the tumors was then compared with those of the untreated controls and tumor growth was graded according to the following scheme: 1) Inconclusive, in groups of animals where 3 or more died, 2) No effect, when the largest diameter was ¾ or more of the diameter of the control tumors, 3) Moderate effect, when the diameter was ¼ to ¾ of the diameter of the control tumors, 4) Marked inhibition when the diameter was ¼ or less of that of the control tumors; actually,

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* We wish to acknowledge support of this study by funds from the American Cancer Society.

[1] Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., Jr., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

[2] Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, **2**, 113.

[3] Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, 1949, **177**, 621.

† A-Methopterin is the name given to the compound by Lederle Laboratories, to whom we are indebted for supplies. The Calco Division, American Cyanamid Company also has provided a supply of this compound.

Procedure and results. One hundred and eighty-seven male mice of the Webster strain were selected for the present experiment at 21 to 23 days of age and an average weight of 10.7 g. The basal ration employed consisted of sucrose 53%, casein[†] 30%, cottonseed oil 10%, Sure's Salt Mixture No. 1[6] 5%, and cellulose[‡] 2%. To each kg of the above diet were added the following synthetic vitamins: thiamine hydrochloride 20 mg, riboflavin 20 mg, pyridoxine hydrochloride 20 mg, nicotinic acid 60 mg, calcium pantothenate 60 mg, biotin 4 mg, folic acid 10 mg, p-aminobenzoic acid 200 mg, inositol 400 mg, 2-methylnaphthoquinone 10 mg, and choline chloride 2 g. Each mouse also received once weekly a vitamin A-D concentrate[§] containing 25 U.S.P. units of vitamin A and 2.5 U.S.P. units of vitamin D together with 1.5 mg of α-tocopherol acetate. The following supplements were incorporated in the basal ration in place of an equal amount of sucrose: (1) 10% yeast[‖] (2) 10% whole liver powder[¶] (3) 10% extracted liver residue,[**] consisting of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances and (4) 2% of Wilson's liver concentrate 1-20,[††] containing the water-extractable material of raw liver. Each of the above diets was in turn supplemented with U.S.P. desiccated thyroid,[‡‡] thyroxin,[§§] or iodinated casein,[‖‖] added in place of an equal amount of sucrose. These were incorporated in the above rations at the following levels: thyroid, 0.5%; thyroxin, 50 mg/kg of diet, and iodinated casein, 0.2%. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib*. Feeding was continued for 15 days or until death, whichever occurred sooner. Animals that died or lost weight during the first 5 days of feeding were discarded and not included in the tabulation of data. Results are summarized in Table I.

The findings indicate the response of the immature mouse is similar to that of the rat when fed massive doses of thyroactive materials. The addition of desiccated thyroid, thyroxin or iodinated casein to the basal ration resulted in a marked retardation of growth which was completely counteracted by the administration of whole liver powder or extracted liver residue. Liver concentrate 1-20 failed to promote growth in animals fed desiccated thyroid or iodinated casein; it did exert a growth-promoting effect, however, in animals fed thyroxin. Yeast was ineffective in promoting growth on any of the diets employed. Data on survival correspond to those obtained in the rat. Less than 50% of the mice fed the basal ration supplemented with desiccated thyroid, thyroxin or iodinated casein survived the experimental period of 15 days. The incidence of survival was significantly increased on diets containing whole liver powder or extracted liver residue. Yeast and liver concentrate 1-20 were considerably less active than whole liver or extracted liver residue in prolonging survival in the hyperthyroid mouse.

In the present experiment whole liver powder prolonged survival and counteracted the growth retardation of immature mice fed massive doses of desiccated thyroid, thyroxin, or iodinated casein. The protective factor(s) was apparently distinct from any of the known nutrients and was retained in the water-insoluble extracted liver residue. Bosshardt *et al.*[7] have recently found that the

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[†] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[6] Sure, B., *J. Nutrition*, 1941, **22**, 449.

[‡] Ruffex, Fisher Scientific Co., St. Louis, Mo.

[§] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per g.

[‖] Brewers' Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

[¶] Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

[**] Extracted Liver Residue, Wilson Laboratories, Chicago, Ill.

[††] Liver Concentrate Powder 1-20, Wilson Laboratories, Chicago, Ill.

[‡‡] Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

[§§] Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J. The material was dissolved in .1 N NaOH, adjusted to pH of 8.0, and diluted to a volume containing 50 mg in 12.5 cc.

[‖‖] Protamone, Cerophyl Laboratories, Kansas City, Mo.

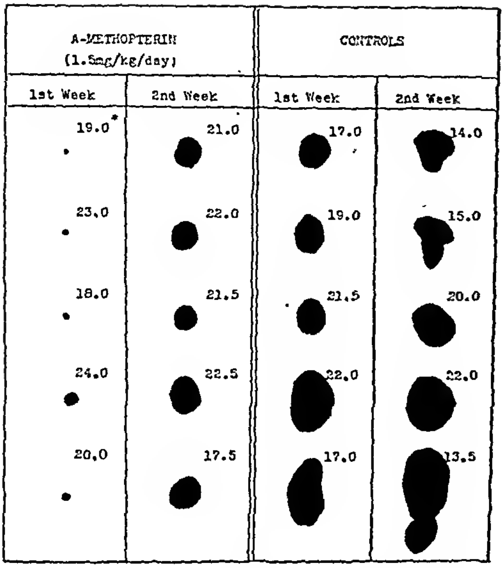

FIG. 1.
Area diagrams of tumors in mice treated with A-Methopterin compared with untreated controls.
* Weight of mouse in grams.

similar effect; however, marked inhibition could be demonstrated only when the drug was administered in amounts which killed a large per cent of the mice. *A-Methopterin* appears to exhibit a more favorable therapeutic index. It has been outstanding in its effect among numerous compounds which might interfere with the utilization of folic acid or possibly with the synthesis of nucleic acid. In fact, it has been the most effective compound of over 700 miscellaneous compounds that have been screened. It is therefore advanced as a compound worthy of further experimental study and for clinical trial *in various types of cancer.*

Summary. The compound 4-Amino-N^{10}-methyl pteroylglutamic acid, has shown the ability to inhibit the growth of the transplantable mouse Sarcoma 180 when administered *intraperitoneally* in concentrations of *1.5 mg/kg each day for 7 days.* At this dosage very few mice died from drug toxicity. The inhibition is most apparent when the compound is administered early in the development of the tumor.

16940

Beneficial Effects of Liver on Growth and Survival in the Immature Hyperthyroid Mouse.*

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Available data indicate that desiccated whole liver will prolong survival and counteract the growth retardation of immature rats fed massive doses of thyroactive materials.[1-5] The protective factor is distinct from any of the known nutrients and is retained in the water-insoluble fraction of liver.[3-5] The present experiment was undertaken to determine the effects of liver and liver fractions on growth and survival in the immature hyperthyroid mouse.

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* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 241 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army. Communication No. 204 from the Department of Biochemistry and Nutrition, University of Southern California.

[1] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 500.

[2] Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, 34, 431.

[3] Ershoff, B. H., *Arch. Biochem.*, 1947, 15, 365.

[4] Ershoff, B. H., *Exp. Med. Surg.*, 1948, 6, 438.

[5] Ershoff, B. H., and McWilliams, H. B., *Science*, 1948, 108, 632.

min B_{12}) failed to counteract the growth retardation of immature mice fed massive doses of desiccated thyroid, thyroxin or iodinated casein while extracted liver residue was as potent as whole liver powder in this regard, it appears that extracted liver residue contains one or more factors other than vitamin B_{12} which are also required in increased amounts by the hyperthyroid mouse. These findings are in agreement with those previously reported for the rat.[4] When animals on purified rations are made hyperthyroid, a multiple dietary deficiency may be precipitated. Whether the first limiting factor will be a deficiency of vitamin B_{12}, of the antithyrotoxic factor(s) of extracted liver residue or of still other nutrients appears to depend on such factors as the pre-test dietary regime, the composition of the diet employed, bacterial synthesis or strain and species differences in nutritional requirements.

Summary. Whole liver powder prolonged survival and counteracted the growth retardation of immature mice fed massive doses of desiccated thyroid, thyroxin or iodinated casein. The protective factor(s) was retained in the water-insoluble fraction of liver.

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[8] Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

[9] Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 40.

16941

An Evaluation of Respiratory Depression by Alveolar Gas Changes During Pentothal Sodium Anesthesia.*

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Recent studies of intravenous anesthetic agents, notably pentothal sodium, have placed renewed emphasis on quantitative analyses of physiological changes occurring during anesthesia. The work of Gruber,[1] Gruhzit, Dox, Rowe and Dodd,[2] Beecher and Moyer,[3,4] Moyer,[5] and most recently McCann,[6] contain pneumographic tracings which illustrate the characteristic respiratory patterns produced by intravenous anesthetic agents. Beecher and Moyer[4] have correlated these qualitative studies with minute volume determinations and intermittent blood levels of oxygen and carbon dioxide. Etsten and Himwich[7,8] intensively investigated the reflex responses under pentothal anesthesia and have set forth criteria for determining the stages of pentothal anesthesia. Previous studies have employed

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* Work done under contract with the Air Materiel Command, Wright Field.

† Present address: Department of Anesthesia, Bellevue Hospital, New York.

[1] Gruber, C. M., *J. Pharm. and Exp. Therap.*, 1937, **60**, 143.

[2] Gruhzit, O. M., Dox, A. W., Rowe, L. W., and Dodd, M. C., *J. Pharm. and Exp. Therap.*, 1937, **60**, 125.

[3] Beecher, H. K., and Moyer, C. A., *J. Clin. Invest.*, 1941, **20**, 549.

[4] Moyer, C. A., and Beecher, H. K., *J. Clin. Invest.*, 1942, **21**, 429.

[5] Moyer, C. A., *J. Thoracic Surg.*, 1941, **11**, 131.

[6] McCann, J. C., *Current Res. in Anesth.-Anal.*, 1947, **26**, 89.

[7] Himwich, H. E., and Etsten, B., *J. Nerv. and Mental Disease*, 1946, **104**, 407.

[8] Etsten, B., and Himwich, H. E., *Anesthesiology*, 1946, **7**, 536.

TABLE I.
Comparative Effects of Liver and Yeast on Growth and Survival of Immature Mice Fed Massive Doses of Thyroactive Substances.

Supplement to basal ration	Thyroactive supplement	No. of animals	Initial body wt, g	Gain in body wt on following days of exp. 10th, g	15th, g	% surviving*
0	0	12	10.6	6.2 (12)	8.1 (12)	100.0
0	Thyroid	15	10.6	2.6 (5)	5.4 (1)	6.7
10% yeast	"	10	10.7	2.9 (8)	5.2 (6)	60.0
10% whole liver powder	"	14	10.5	7.7 (14)	11.2 (13)	92.9
10% ext. liver residue	"	15	10.4	6.6 (15)	10.0 (15)	100.0
1% liver conc. 1-20	"	15	10.5	3.3 (9)	3.8 (6)	40.0
0	Iodinated casein	12	10.9	2.8 (10)	4.2 (4)	33.3
10% yeast	" "	10	11.0	2.8 (10)	5.1 (6)	60.0
10% whole liver powder	" "	12	10.7	8.4 (11)	11.9 (11)	91.7
10% ext. liver residue	" "	9	11.1	7.3 (8)	9.7 (8)	88.9
1% liver conc. 1-20	" "	8	11.1	2.0 (8)	3.6 (6)	75.0
0	Thyroxin	11	10.7	1.0 (10)	2.6 (5)	45.5
10% yeast	"	9	10.8	3.6 (6)	4.3 (6)	66.7
10% whole liver powder	"	11	10.8	5.3 (11)	8.4 (9)	81.8
10% ext. liver residue	"	12	10.6	8.9 (11)	12.8 (11)	91.7
1% liver conc. 1-20	"	12	10.9	6.4 (7)	9.8 (6)	50.0

The values in parentheses indicate the number of animals which survived and on which averages are based.

* Experimental period 15 days.

growth retardation of immature mice fed massive doses of iodinated casein (protamone) could be counteracted by the administration of Wilson's liver concentrate 1-20 and other water-soluble extracts of liver rich in "animal protein factor" or vitamin B_{12}. Under conditions of the present experiment, Wilson's liver concentrate 1-20 contained virtually no activity in regard to either growth or survival for immature mice fed massive doses of iodinated casein or desiccated thyroid. It did exert a growth-promoting effect, however, in immature mice fed thyroxin, although gain in body weight was less than that obtained with extracted liver residue. The cause of this discrepancy is not readily apparent. Yeast appears to be less beneficial to the hyperthyroid mouse than to the hyperthyroid rat. Previous findings indicate that yeast is as effective as whole liver powder in prolonging survival of immature rats fed massive doses of desiccated thyroid;[3] such does not appear to be the case for the immature thyroid-fed mouse.

Recent findings by Bosshardt *et al.*,[7] Register *et al.*,[8] and Nichol *et al.*,[9] indicate that requirements for vitamin B_{12} are increased in the hyperthyroid animal. Since liver concentrate 1-20 (which contains vitarecovered to the point of restlessness, or for a period of 30 to 55 minutes. Maximum respiratory depression was reached in approximately 10 minutes and a gradual return to normal respiratory values followed. Signs of awakening and return of reflexes occurred as the respiratory picture of the animals approached control levels.

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[7] Bosshardt, D. K., Paul, W. J., O'Doherty, K., Huff, J. W., and Barnes, R. H., *J. Nutrition*, 1949, 37, 21.

A typical record is shown in Fig. 1. It can be seen that the period of maximum depression is attained about 10 minutes after injection. This depression period is characterized by the lowest ventilation volume with its concomitant low pO_2, R.Q. and high pCO_2. The last period of recovery is associated with relative hyperventilation and an R.Q. above normal.

2. Intermittent injection of fractional doses of pentothal sodium.

Two dogs were given intermittent injections of approximately 0.25 g of pentothal sodium until a total of 1 g was administered. These experiments were carried on in precisely the same manner except that the recordings extended over a period of 140 minutes. The same general pattern of Fig. 1 and 2 repeated itself following each intermittent injection.

3. Intermittent injection of pentothal sodium in patients undergoing elective operative procedures.

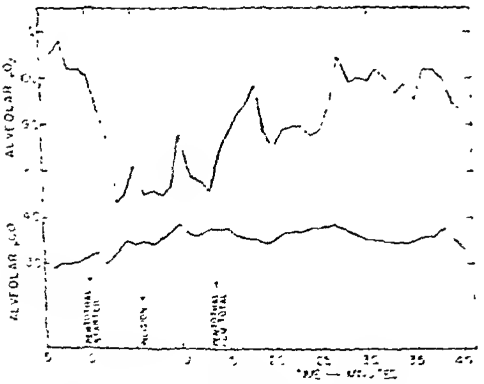

FIG. 2.

Alveolar pO_2 and pCO_2 recorded continuously in a patient before, during and after receiving pentothal sodium intermittently over a 14-minute period. Total dose 1 g. The fluctuations, particularly in the pO_2 level, arise from reflex hyperventilation.

Nine patients undergoing operative procedures were observed. Attempts to obtain control levels of alveolar air in the immediate preoperative period were not totally successful because of the apprehensive state of mind of the patient and the disturbances caused by preoperative medication. During light pentothal anesthesia operative stimulation always produced marked reflex hyperventilation which is reflected in the recorded values (Fig. 2). However, these responses are superimposed upon the respiratory pattern described above as characteristic of pentothal anesthesia in dogs.

Discussion. The question arises of a suitable quantitative criterion for the measurement of respiratory depression. If the total ventilation is used as an index one may make two errors. The first error is produced by changes in the oxygen consumption from the resting, conscious state to the anesthetized state. Thus a reduction of 30% of ventilation is without effect upon the alveolar gas concentration if likewise the oxygen consumption drops by the same amount. The second error is encountered when the breathing rate alters the dead space ventilation. For example a total ventilation of 6 liters per minute may break down into 4 liters alveolar ventilation and 2 liters dead space ventilation (10 respirations of a 200 cc dead space). The same total ventilation can be achieved by 20 respirations of the same dead space and a 2 liter alveolar ventilation. If the former produces a normal alveolar gas composition the latter situation would produce hypoxia and severe acidosis.

Thus unless these entities are known or can be measured the total ventilation may be a poor quantitative index of respiratory depression or stimulation. This is particularly so if one is interested in the effects of the altered ventilation upon the blood and tissue gas tensions.

The next best alternative is to measure *absolute alveolar ventilation* which eliminates the dead space ventilation but is dependent upon the oxygen consumption and for this reason is still unsatisfactory.[12] If respiratory depression or stimulation must be expressed the measurement of total ventilation volume as an index of respiratory depression or stimulation. As will be discussed below such measurements may lead to erroneous conclusions when applied quantitatively. Instead, it is here proposed to describe the degree of the effective ventilation in terms of the alveolar gas concentration only, which makes the consideration of volume measurements unnecessary. This method has the further advantage that the alveolar gas concentrations reflect the gas pressures of the arterial blood if corrected for the alveolar-arterial gradient. Suskind[9] has recently shown that during anesthesia this gradient, at least for CO_2, is nonexistent. Thus changes which occur in the pCO_2 of the alveolar air allow one to calculate changes in the arterial pH if the plasma bicarbonates are known.

Method. Alveolar air was automatically sampled by a simple device described by Rahn and Otis[10] and continuously analyzed as previously reported.[11] Readings were made at one minute intervals and the respiratory quotient calculated from the alveolar air equation (Fenn, Rahn, and Otis).[12]

Ventilation volume. The air was expired through a gas meter. Each passing of 457 cc of gas produced an electrical contact which closed the circuit of a magnetic writing pen recording on a constant-speed paper tape.

Frequency of respiration was recorded on the same tape by another pen. The slight negative mask pressure during each inspiration activated a sensitive aneroid which in turn closed the electrical circuit.

Three dogs each weighing approximately 23 kilos were fitted with individually constructed skin-tight masks. After an initial training period alveolar air could be sampled in the unanesthetized animal. Pentothal sodium was then injected into the saphenous vein and all the respiratory phenomena were recorded without interruption. After losing consciousness the dogs were placed in the lateral recumbent position with the head in hyperextension for the remainder of the experiment. Specific attention was placed on the maintenance of an adequate, unobstructed airway by position of the head.

Alveolar gas analyses were also performed in the operating pavilion on 9 patients undergoing elective operative procedures under pentothal anesthesia administered by an intermittent injection technic.

Results. 1. Single injection of 0.5 g of pentothal sodium in dogs.

Three dogs were each given a single injection of 0.5 g of pentothal over a 2-5 minute period with mask in place while continuous analyses were being carried out. Observations were continued until the animal had

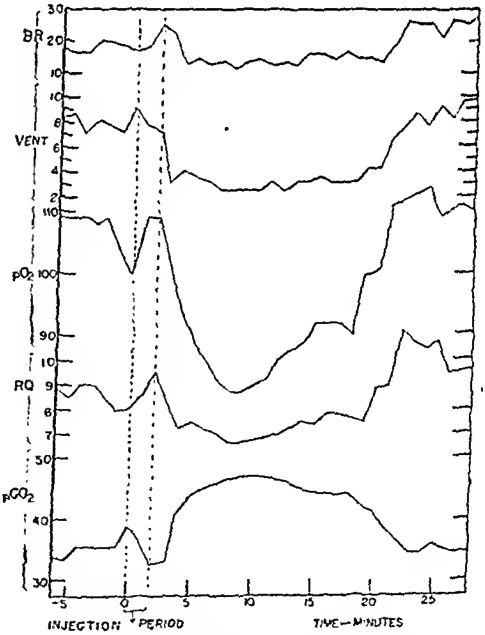

FIG. 1.

Respiratory responses of dog S to 0.5 g pentothal sodium. Injection occurred between 0 and 2 minutes. BR, breaths per minute; Vent, liter per minute expired at B.T.P.S. (body temperature, pressure and saturated): pO_2 and pCO_2 mm Hg alveolar pressure of the gases; R.Q., alveolar respiratory quotient.

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[9] Suskind, M., unpublished.

[10] Rahn, H., and Otis, A. B., *J. Applied Physiol.*, 1949, **1**, No. 10.

[11] Rahn, H., Mohney, J., Otis, A. B., and Fenn, W. O., *J. Aviation Med.*, 1946, **17**, 173.

[12] Fenn, W. O., Rahn, H., and Otis, A. B., *Am. J. Physiol.*, 1946, **146**, 637.

recovered to the point of restlessness, or for a period of 30 to 55 minutes. Maximum respiratory depression was reached in approximately 10 minutes and a gradual return to normal respiratory values followed. Signs of awakening and return of reflexes occurred as the respiratory picture of the animals approached control levels.

A typical record is shown in Fig. 1. It can be seen that the period of maximum depression is attained about 10 minutes after injection. This depression period is characterized by the lowest ventilation volume with its concomitant low pO_2, R.Q. and high pCO_2. The last period of recovery is associated with relative hyperventilation and an R.Q. above normal.

2. Intermittent injection of fractional doses of pentothal sodium.

Two dogs were given intermittent injections of approximately 0.25 g of pentothal sodium until a total of 1 g was administered. These experiments were carried on in precisely the same manner except that the recordings extended over a period of 140 minutes. The same general pattern of Fig. 1 and 2 repeated itself following each intermittent injection.

3. Intermittent injection of pentothal sodium in patients undergoing elective operative procedures.

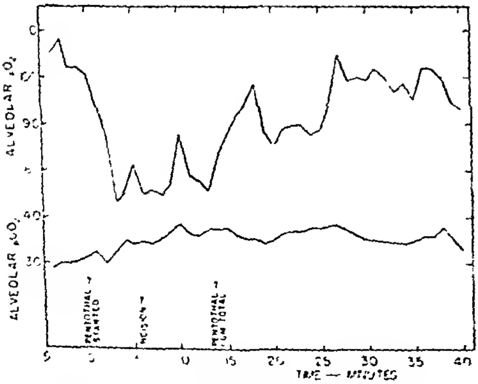

FIG. 2.

Alveolar pO_2 and pCO_2 recorded continuously in a patient before, during and after receiving pentothal sodium intermittently over a 14-minute period. Total dose 1 g. The fluctuations, particularly in the pO_2 level, arise from reflex hyperventilation.

Nine patients undergoing operative procedures were observed. Attempts to obtain control levels of alveolar air in the immediate preoperative period were not totally successful because of the apprehensive state of mind of the patient and the disturbances caused by preoperative medication. During light pentothal anesthesia operative stimulation always produced marked reflex hyperventilation which is reflected in the recorded values (Fig. 2). However, these responses are superimposed upon the respiratory pattern described above as characteristic of pentothal anesthesia in dogs.

Discussion. The question arises of a suitable quantitative criterion for the measurement of respiratory depression. If the total ventilation is used as an index one may make two errors. The first error is produced by changes in the oxygen consumption from the resting, conscious state to the anesthetized state. Thus a reduction of 30% of ventilation is without effect upon the alveolar gas concentration if likewise the oxygen consumption drops by the same amount. The second error is encountered when the breathing rate alters the dead space ventilation. For example a total ventilation of 6 liters per minute may break down into 4 liters alveolar ventilation and 2 liters dead space ventilation (10 respirations of a 200 cc dead space). The same total ventilation can be achieved by 20 respirations of the same dead space and a 2 liter alveolar ventilation. If the former produces a normal alveolar gas composition the latter situation would produce hypoxia and severe acidosis.

Thus unless these entities are known or can be measured the total ventilation may be a poor quantitative index of respiratory depression or stimulation. This is particularly so if one is interested in the effects of the altered ventilation upon the blood and tissue gas tensions.

The next best alternative is to measure *absolute alveolar ventilation* which eliminates the dead space ventilation but is dependent upon the oxygen consumption and for this reason is still unsatisfactory.[12] If respiratory depression or stimulation must be expressed

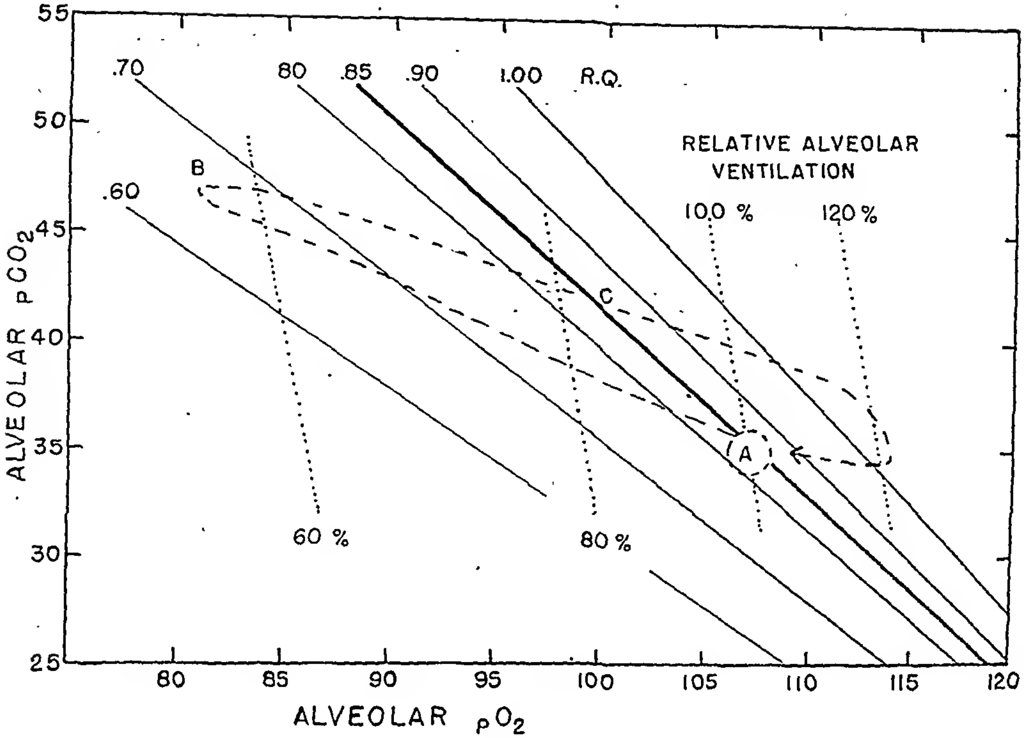

FIG. 3.

Typical alveolar pathway of dog S to single injection of pentothal sodium indicating the simultaneous alveolar pO_2, pCO_2, R.Q., and relative alveolar ventilation. Point A is the average, unanesthetized level prior to injection. Region B indicates the period of deepest depression, region B-C the period of compensation, and C-A the period of recovery characterized by high R.Q. For details see discussion.

in terms of ventilation volume it probably can be most satisfactorily done by calculating the *relative alveolar ventilation* from the alveolar gas concentration. This new derivation is discussed below.

On the other hand the difficulties in the foregoing discussion may be entirely avoided if respiratory depression or stimulation is measured in terms of the deviation it induces from the normal alveolar CO_2 and O_2. Thus the net result of the effective ventilation is expressed without involving the measurement of a ventilation volume. In Fig. 1 the various recorded values are plotted against time. In Fig. 3 the alveolar O_2 and CO_2 are plotted against each other and the whole period of anesthesia is represented by a point moving in a clockwise direction. Each point on such a graph simultaneously determines the alveolar respiratory quotient as well as the relative ventilation index. The iso-R.Q. lines are straight and originate at the inspired O_2 tension while the iso-ventilation lines have a negative slope of .209 when air is inspired.[12] The advantage of this type of graph is that it allows the simultaneous visualization of the various components of respiration.

Point A in Fig. 3 is the average alveolar air composition of the unanesthetized dog just prior to the injection of pentothal. The R.Q. is .85 and the alveolar ventilation is assumed to be normal or 100%. Following the injection, respiration is depressed. This is seen by a rapid fall in the alveolar pO_2 and R.Q. and a slower rise of the pCO_2 until point B is reached. The curve from A to B is very constant in dog and man and has been designated as the "respiratory acidosis pathway" by Rahn and Otis.[10] It is reproducible from time to time and has been observed whenever the alveolar ventilation is reduced by dead space breathing, anesthesia or voluntary apnea.[10,13]

Point B indicates the deepest depression reached about 10 minutes after the injection and represents the greatest retention of metabolic CO_2 as indicated by the low R.Q. value. Since Suskind[9] has recently shown in this laboratory that there is no significant difference in the anesthetized dog between the arterial and alveolar pCO_2 one may estimate the changes in arterial pH accompanying the pCO_2 rise. Assuming a normal base bicarbonate level of the plasma the Henderson-Hasselbalch equation indicates a pH drop of .12 units between point A and B. Unpublished studies with standard doses of nembutal show much greater depression yielding CO_2 and O_2 values of 50 and 60 mmHg respectively.

This initial respiratory depression is followed by a period of compensation. The CO_2 stores of blood and tissues are slowly adjusted to the new alveolar CO_2 tension and as the rate of accumulation of CO_2 decreases, the R.Q. returns to its original value (Point C). However, the new steady state is not maintained. Throughout the period of compensation, the anesthetic is wearing off, and the sensitivity of the respiratory system to CO_2 is increasing. Thus we see during the final stages of recovery (region C-A) a relative hyperventilation which rids the blood and tissues of the recently acquired CO_2 stores and is manifested by an R.Q. above .85. The 3 respiratory phases described here, depression A-B, compensation B-C, and recovery C-A, all grade into one another and last approximately 10 minutes each. With complete recovery the CO_2 tension in blood, tissues and alveoli are readjusted to the original value of point A and the steady state with a normal R.Q. is observed.

At this time the dog becomes restless, and when an additional injection is given the whole cycle repeats itself. This was accomplished 3 times in succession in one dog and twice in another, the total period of anesthesia lasting over a period of 2 hours. This alveolar pathway cycle is typical not only for anesthesia but also in conscious man when the alveolar ventilation is reduced by various artificial means.

Should it be desirable to express the drug action in terms of a ventilation unit this can be done by assuming that the ventilation is normal (100%) only when it maintains an alveolar concentration of point A, the control value during the conscious state. This unit is called the *relative alveolar ventilation,* which has the advantage that it makes ventilation independent of changes in metabolic rate and thus offers a better physiological comparison for fluctuating metabolic rates as encountered in anesthetic studies.

The *relative alveolar ventilation* of point A is equal to $\frac{\text{k. R.Q. } 100}{\text{alv. } pCO_2}$ where k is a constant yielding a ventilation of 100% when the values of point A are substituted. (This equation is derived from the alveolar ventilation equation, Fenn[12]). The constant for our example is 41.2. When lines of equal "relative alveolar ventilation" are plotted they form straight lines as indicated in Fig. 3.

It can be seen that the deepest respiratory depression is obtained when the alveolar ventilation is 58% of the ventilation required to maintain the alveolar air composition at point A. Recovery, on the other hand, is associated with a relative hyperventilation reaching a maximal value just over 120%. Fig. 1, however, indicates a much larger relative change in the *total ventilation,* (from 100% to 38%) and is therefore misleading unless the marked drop in oxygen consumption and breathing rate is properly evaluated.

It is obvious from the data that respiratory stimulation or depression is a continuously changing state. This makes a quantitative comparison difficult unless some particular phase of the anesthesia cycle is employed as reference. Since *total ventilation* as an index may be quite misleading it is here suggested that the deviation from the normal alveolar pCO_2, R.Q. and/or *relative alveolar ventilation* be used as a quantitative and physiologically comparable approach to studies of res-

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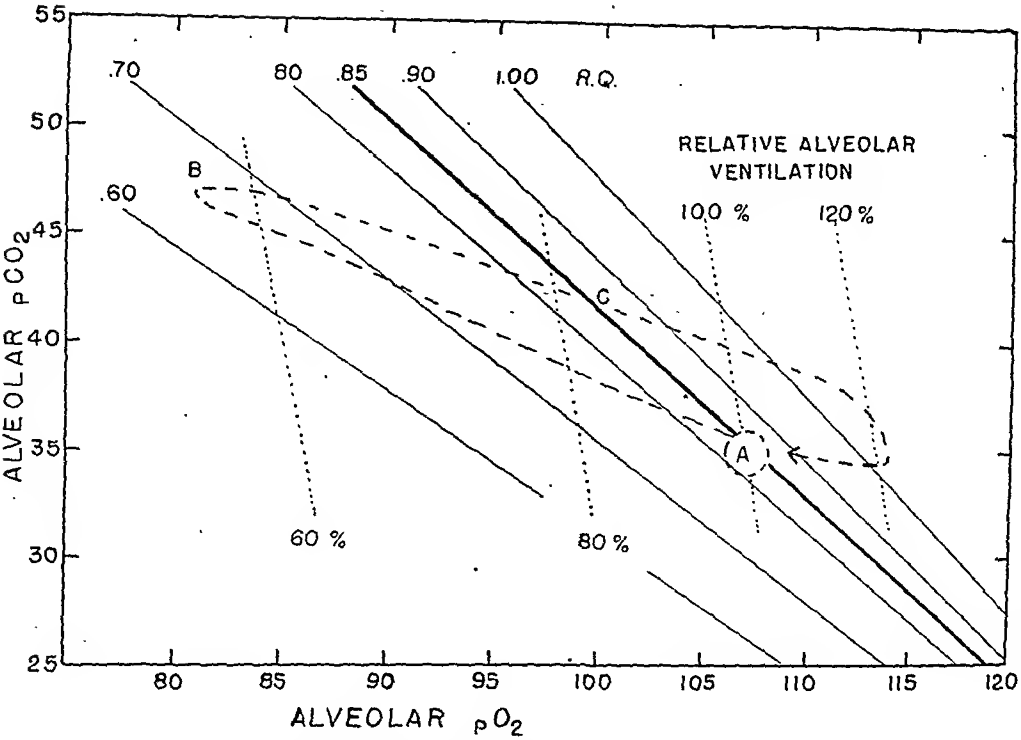
[13] Otis, A. B., Rahn, H., and Fenn, W. O., *Am. J. Physiol.*, 1948, **152**, 674.



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3. With intermittent injections of pentothal sodium this typical pattern is repeated.

4. The theoretical significance of this respiratory pattern and its utilization for analysis of quantitative changes in respiration in the study of anesthetic agents is discussed.

16942

A Technic for the Recovery of Viruses from Crude Feces.

HERBERT R. MORGAN.*

From the Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Mich.

During the course of studies on the isolation of psittacosis and other viruses from feces, an attempt was made to develop a method, which would allow for direct inoculation of a crude fecal suspension into eggs, for the recovery of a variety of viruses by the use of anti-bacterial agents. Several different test agents were used to determine the applicability of the technic to known viruses in the hope that it might prove useful in the isolation of as yet unknown viruses from the gastrointestinal tract of man and animals. Since penicillin and the sulfonamides are known to inhibit certain viruses of the psittacosis-lymphogranuloma venereum group, they were eliminated from consideration.[1,2] Streptomycin has been shown to have no effect on them[1] nor on several other virus agents[3-6] so that it was useful for study. However, streptomycin alone is not efficacious against all gram positive organisms[7,8] so that it was necessary to find another agent to aid in the suppresion of the gram positive organisms in the feces.

The anti-bacterial agents tested were streptomycin, tyrothrycin in alcoholic solution, tyrothrycin in propylene glycol solution and crystal violet.

Since specimens are frequently submitted for virus isolation studies from patients receiving sulfonamide and/or penicillin therapy, it was of interest to determine whether psittacosis virus strains, known to be susceptible to their action, could be recovered from virus-fecal suspensions containing sulfadiazine or penicillin by the use of antagonistic or inactivating compounds such as p-aminobenzoic acid or cysteine hydrochloride.

The yolk sac route was chosen for the

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[2] Meiklejohn, G., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 1.

[3] Morgan, H. R., and Wiseman, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 130.

[4] Florman, A. L., Weiss, A. B., and Council, F. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 16.

[5] Lowell, F. C., and Buckingham, M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 228.

[6] Henle, G., Henle, W., Wendell, K. K., and Rosenberg, P., *J. Exp. Med.*, 1948, **88**, 223.

[7] Hodges, J. H., *Science*, 1946, **104**, 460.

[8] Rose, H. M., Pearce, E., and Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 124.

inoculation of eggs since (a) it allows for inoculation of a large volume of material and (b) it has been shown to be the route of choice for the inoculation of psittacosis virus as well as an excellent method for the cultivation of a wide variety of viruses.[9-11]

Three viruses were selected as models for this study: psittacosis (strain 6 BC), influenza A (PR8) and mumps.

Material and methods. A 10% suspension of human feces in infusion broth was prepared by homogenization in a Waring blendor. Large sized particles were removed by centrifugation at 800 rpm for 3 minutes. The suspension had a bacterial plate count of 2.1×10^7 organisms per ml. An aliquot of the fecal suspension was sterilized in the autoclave. To separate aliquots of the fresh fecal suspension and of the sterile fecal suspension, an active preparation of one virus was added to give a virus dilution of 1:10. Each different suspension was placed in a separate series of glass ampoules, sealed and stored in the dry icebox. The virus preparations added were: (a) a suspension of yolk sacs infected with psittacosis virus, or (b) allantoic fluid infected with PR8 influenza virus, or (c) allantoic fluid infected with mumps virus.

In testing each of the viruses with a diluent containing different types and amounts of inhibiting agents, serial 10 fold dilutions of the following were prepared:

(a) Virus-sterile feces diluted in plain beef heart infusion broth

(b) Virus-sterile feces diluted in beef heart infusion broth containing the antibacterial agents

(c) Virus-fresh feces diluted in plain beef heart infusion broth

(d) Virus-fresh feces diluted in beef heart infusion broth containing the antibacterial agents

The dilutions were allowed to stand at room temperature (*i.e.* 20–24°C) for 1-2 hours following which 0.25 ml amounts of each were injected into the yolk sacs of 12 embryonated eggs 6 or 7 days old. The eggs were placed in an incubator at 35°C and were candled daily. Eggs inoculated with 10 fold dilutions of the virus + fresh feces in plain broth up through 10^{-6} died within 24-72 hours with gross evidence of contamination while eggs inoculated with the suspension diluted in broth containing the inhibitors died in from 2 to 10 days depending on the particular virus and the degree of its dilution. As the latter group of eggs died: (a) smears were made of the yolk sacs, stained and examined to detect bacteria, and, in the case of eggs injected with psittacosis virus, were stained by Machiavello's method to demonstrate the typical elementary bodies, (b) in some instances, the yolk sacs were cultured on blood agar plates and in thioglycollate broth to test the reliability of the smears as an indicator of bacterial contamination, (c) in the case of the eggs inoculated with suspensions containing influenza or mumps viruses, the yolk, allantoic and amniotic fluids of the egg were tested for their ability to agglutinate chicken red cells as an index of the presence of virus.

As eggs in the group inoculated with the virus-sterile feces suspension diluted in plain broth or broth containing the bacteriostatic agents died, they were tested as noted above for the presence of the specific virus injected. These two groups served as controls for any possible deleterious action of the bacteriostatic agents on the virus itself.

In order to see whether virus could be recovered from specimens containing penicillin or sulfonamides, the following experiment was carried out: (a) 1000 U penicillin were injected into each egg of a series along with the suspension of the virus in sterile feces. Then 15 *mg* of cysteine hydrochloride[12] were injected into different groups of these eggs at intervals up to 24 hours. (b) A second series of eggs was injected with a suspension

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[9] Morgan, H. R., Early, R. L., and McClain, M. E., *J. Inf. Dis.*, 1946, 79, 278.

[10] Morgan, H. R., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1948, 68, 618.

[11] Beveridge, W. I. B., and Burnet, F. M., Special Report Series No. 256, Medical Research Council, His Majesty's Stationery Office, London, 1946.

[12] Chow, B. F., and McKee. C. M., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 175.

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16943

Lupulon—An Antibiotic Extracted from the Strobiles of *Humulus lupulus*.*†

A. J. SALLE, GREGORY J. JANN, AND MICHAEL ORDANIK.
From the Department of Bacteriology, University of California, Los Angeles.

Lupulon is a nearly white, crystalline substance obtained by fractionating the resins extracted with methanol and other solvents from the strobiles of the hop *Humulus lupulus*.

According to Taylor and Millidge[1] lupulon possesses the following properties: It has a melting point of 92°C, is unsaturated, adds bromine, decolorizes permanganate, and has the properties of a keto-enol.

Walker and Parker[2] reported that the hop resins exhibited antibacterial properties. Shimwell[3] found that lupulon inhibited the growth of Gram-positive bacteria but had no effect on Gram-negative organisms.

The present communication is concerned with a study of the bacteriologic and mycologic properties of lupulon *in vitro* and its use in the treatment of bacterial infections in mice.

Materials and methods. A solution of lupulon was prepared by dissolving one gram in 25 ml of propylene glycol, heating slightly, then adding sufficient distilled water to make a 1:500 opalescent emulsion of the resinous antibiotic.

The bactericidal (with the exception of *Mycobacterium tuberculosis*) and fungicidal spectra were determined by the penicylinder method using a medium having the following composition: Sodium chloride (NaCl), 5 g; beef extract (Bacto), 5 g; peptone (Bacto), 10 g; agar (Bacto), 20 g; and distilled water, to make 1000 ml. The medium was adjusted

TABLE I.
Effect of Lupulon on a Number of Bacteria by the Penicylinder Method.

Gram-positive bacteria 1:10,000 dilution of lupulon	Results	Gram-negative bacteria 1:500 dilution of lupulon	Results
Micrococcus lysodeikticus	—	*Aerobacter aerogenes*	+
'' *pyogenes* var. *aureus*	—	*Alcaligenes faecalis*	+
'' *ureae*	—	'' *viscosus*	+
Bacillus anthracis	—	*Escherichia coli*	+
'' *megatherium*	—	'' *coli* var. *communior*	+
'' *subtilis*	—	*Klebsiella pneumoniae*	+
Gaffkya tetragena	—	*Proteus vulgaris*	+
Rhodococcus roseus	—	*Pseudomonas aeruginosa*	+
Sarcina lutea	—	'' *synxantha*	+
'' *ureae*	—	*Salmonella enteritidis*	+
Streptococcus faecalis	—	'' *paratyphi A*	+
'' *lactis*	—	'' *schottmuelleri*	+
		'' *typhosa*	+
		Serratia marcescens	+
		Shigella ambigua	+
		'' *dysenteriae*	+
		'' *paradysenteriae*	+
		'' *sonnei*	+

+ = growth; — = no growth.

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* This work was supported in part by a grant-in-aid from the Antibiotics Study Section, U. S. Public Health Service, National Institute of Health, Bethesda, Md.

† The lupulon used in these experiments was kindly supplied by the Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, Calif.

[1] Taylor, T. W. J., and Millidge, A. F., Richter's Organic Chemistry, Vol. II, Nordeman Publishing Co., Inc., New York, 1939.

[2] Walker, T. K., and Parker, A., *J. Inst. Brew.*, 1937, **34**, 17.

[3] Shimwell, J. L., *J. Inst. Brew.*, 1937, **34**, 111, 191.

of virus in sterile feces along with 10 mg of sulfadiazine. At intervals up to 24 hours, 1 mg of p-aminobenzoic acid[13,14] was injected into different groups of these eggs. In all instances, the eggs died after incubation at 35°C for several days. Smears of the yolk sacs were stained and examined for the presence of psittacosis virus elementary bodies.

Results. Using a diluent of beef heart infusion broth containing 10 mg of streptomycin and 2 mg of tyrothrycin per ml (containing 1% glycerine to give a stable suspension of the alcoholic solution of tyrothrycin added to broth), it was possible to recover the viruses of psittacosis, influenza or mumps which had been added to a fresh suspension of feces without any evidence that the bacteriostatic agents used in the diluent for the suppression of bacterial contaminants had any deleterious effect on the virus. In fact, in many instances, even the eggs injected with a 10^{-1} dilution of the fecal suspension of virus (10^{-2} dilution of virus infected fluid) could be shown to contain virus free of detectable bacteria even though this dilution was shown to contain at least 2.1×10^{6} aerobic bacteria per ml. High dilutions (*i.e.* 10^{-2} or 10^{-3}) almost always gave virus free from bacterial contaminants. This technic was found useful in the isolation of psittacosis virus from the ground-up intestines and feces of mice infected with the virus by the intravenous route.

In some experiments, a solution of tyrothrycin in propylene glycol was used but this solvent was found to inhibit the growth of the viruses. Therefore, the alcoholic solution was used subsequently with the addition of 1% glycerine to stabilize its suspension in broth.

It is important to point out that tyrothrycin can be used as a bacteriostatic agent in materials to be tested in eggs only if the yolk sac route of inoculation is used since it is toxic in 0.2 mg amounts in the allantoic or amniotic sacs.

Crystal violet in amounts up to 0.25 mg was tolerated when injected into the yolk sac of embryonated eggs but was not effective in controlling the gram positive bacteria in fecal suspensions tested.

It was found that the injection of (a) 15 mg of cysteine hydrochloride or (b) 1 mg of p-aminobenzoic acid into the yolk sac any time up to 24 hours after the injection of as much as (a) 1000 U of penicillin or (b) 10 mg of sulfadiazine respectively in the virus-feces inoculum would permit the recovery of psittacosis virus (strain 6 BC) which was known to be sensitive to 250 U of penicillin[1] or 0.5 mg of sulfadiazine.[14]

Summary. Using an infusion broth diluent containing 10 mg streptomycin and 2 mg tyrothrycin per ml, it was possible, by injection into the yolk sacs of 6-7-day-old embryonated eggs, to recover free from bacteria: psittacosis, influenza or mumps viruses from suspensions of fresh feces to which they had been added. The diluent had no apparent injurious effect on these viruses.

P-aminobenzoic acid was found to counteract the inhibitory action of sulfadiazine allowing for the recovery of psittacosis virus when the virus was suspended in materials containing the sulfonamide and injected into eggs. The injection of the p-aminobenzoic acid could be delayed up to 24 hours when injected in 1 mg amounts after a virus suspension containing 10 mg of sulfadiazine. In a similar series of experiments it was found that 15 mg cysteine hydrochloride would overcome the inhibitory action of 1000 U penicillin on psittacosis virus when given as long as 24 hours after the virus suspension containing this antibiotic. These antagonistic compounds made it possible to recover psittacosis virus from materials containing large quantities of sulfadiazine or penicillin in spite of the fact that this strain of psittacosis virus is known to be susceptible to much smaller quantities of each of these therapeutic agents.

This technic was useful in the isolation of virus from the intestines of mice infected with psittacosis virus and may find application in the isolation of viruses from the gastrointestinal tract of other animals or man.

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[13] Morgan, H. R., PROC. SOC. EXP. BIOL. AND MED., 1948, 67, 29.

[14] Morgan, H. R., *J. Exp. Med.*, 1948, 88, 285.

16943

Lupulon—An Antibiotic Extracted from the Strobiles of *Humulus lupulus*.*†

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Lupulon is a nearly white, crystalline substance obtained by fractionating the resins extracted with methanol and other solvents from the strobiles of the hop *Humulus lupulus*.

According to Taylor and Millidge[1] lupulon possesses the following properties: It has a melting point of 92°C, is unsaturated, adds bromine, decolorizes permanganate, and has the properties of a keto-enol.

Walker and Parker[2] reported that the hop resins exhibited antibacterial properties. Shimwell[3] found that lupulon inhibited the growth of Gram-positive bacteria but had no effect on Gram-negative organisms.

The present communication is concerned with a study of the bacteriologic and mycologic properties of lupulon *in vitro* and its use in the treatment of bacterial infections in mice.

Materials and methods. A solution of lupulon was prepared by dissolving one gram in 25 ml of propylene glycol, heating slightly, then adding sufficient distilled water to make a 1:500 opalescent emulsion of the resinous antibiotic.

The bactericidal (with the exception of *Mycobacterium tuberculosis*) and fungicidal spectra were determined by the penicylinder method using a medium having the following composition: Sodium chloride (NaCl), 5 g; beef extract (Bacto), 5 g; peptone (Bacto), 10 g; agar (Bacto), 20 g; and distilled water, to make 1000 ml. The medium was adjusted

TABLE I.
Effect of Lupulon on a Number of Bacteria by the Penicylinder Method.

Gram-positive bacteria 1:10,000 dilution of lupulon	Results	Gram-negative bacteria 1:500 dilution of lupulon	Results
Micrococcus lysodeikticus	—	*Aerobacter aerogenes*	+
'' *pyogenes* var. *aureus*	—	*Alcaligenes faecalis*	+
'' *ureae*	—	'' *viscosus*	+
Bacillus anthracis	—	*Escherichia coli*	+
'' *megatherium*	—	'' *coli* var. *communior*	+
'' *subtilis*	—	*Klebsiella pneumoniae*	+
Gaffkya tetragena	—	*Proteus vulgaris*	+
Rhodococcus roseus	—	*Pseudomonas aeruginosa*	+
Sarcina lutea	—	'' *synxantha*	+
'' *ureae*	—	*Salmonella enteritidis*	+
Streptococcus faecalis	—	'' *paratyphi A*	+
'' *lactis*	—	'' *schottmuelleri*	+
		'' *typhosa*	+
		Serratia marcescens	+
		Shigella ambigua	+
		'' *dysenteriae*	+
		'' *paradysenteriae*	+
		'' *sonnei*	+

+ = growth; — = no growth.

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* This work was supported in part by a grant-in-aid from the Antibiotics Study Section, U. S. Public Health Service, National Institute of Health, Bethesda, Md.

† The lupulon used in these experiments was kindly supplied by the Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, Calif.

[1] Taylor, T. W. J., and Millidge, A. F., Richter's Organic Chemistry, Vol. II, Nordeman Publishing Co., Inc., New York, 1939.

[2] Walker, T. K., and Parker, A., *J. Inst. Brew.*, 1937, **34**, 17.

[3] Shimwell, J. L., *J. Inst. Brew.*, 1937, **34**, 111, 191.

TABLE II.
Effect of Lupulon on a Group of Higher Organisms by the Penicylinder Method.

1:500 dilution of lupulon	Results
Aspergillus flavus	+
,, *niger*	+
Candida albicans	+
Cryptococcus neoformans	+
Discomyces mexicanus	+
Epicoccum nigrum	+
Microsporum lanosum	+
Nocardia asteroides	+
,, *madurae*	+
Penicillium glabrum	+
Rhizopus nigricans	+
Streptomyces pelletieri	+
Trichoderma köningii	+
Trichophyton gypseum	+

+ = growth.

to pH 7.0 and sterilized at 15 lb pressure for 30 minutes.

Proskauer and Beck's medium was used for the growth of *M. tuberculosis.* This is a liquid medium having the following composition: Asparagine, 5 g; potassium acid phosphate (KH_2PO_4), 5 g; potassium sulfate (K_2SO_4), 0.6 g; magnesium citrate soluble ($MgHC_6H_5O_7 \cdot 5H_2O$), 2.5 g; glycerol, 25 g; and distilled water, to make 1000 ml.

Results. The action of lupulon on a number of Gram-positive and Gram-negative bacteria by the penicylinder method is given in Table I. It may be seen that a 1:10,000 dilution of the antibiotic was effective against Gram-positive bacteria but displayed little or no activity against Gram-negative organisms even when used in a concentration as high as 1:500.

In Table II are given the results of lupulon on a group of higher organisms. The antibiotic produced no apparent effect when used in a 1:500 dilution.

Lupulon was tested against several organisms in the presence of 10% horse serum[‡] (Table III). The results show that lupulon exhibited no antibiotic effect when tested in the presence of this agent.

Eight mice were injected with 0.1 cc of a virulent broth culture of *Streptococcus pyogenes.* Four of the mice were treated with lupulon and 4 were used as controls. Doses of 2 mg of the antibiotic injected intraperitoneally every 3 hours failed to produce any demonstrable effect on the outcome of the infection. All animals were dead after 24 hours. It may be concluded that the antibiotic was completely inactivated *in vivo.*

According to Taylor and Millidge[1] lupulon has the structural formula shown in (A), Fig. 1. Lupulon may exist in any of the 3 forms shown but forms (B) and (C) probably predominate because they contain more highly conjugated systems and hence have more resonance of stabilization than (A).

Lupulon is slowly inactivated when in solution. The effect of a freshly prepared and an old solution of lupulon on *M. tuberculosis* H37Rv is shown in Table IV. A freshly prepared solution is considerably more effective than one 10 days old.

Inactivation on standing is compatible with the general instability of the molecule. The side chain in position 5 contains an allylic

TABLE III.
Effect of Lupulon on *Micrococcus pyogenes* var. *aureus* and *Mycobacterium tuberculosis* H37Rv in the Presence and Absence of Horse Serum.

Dilution of lupulon	*M. pyogenes* var. *aureus* With 10% serum	*M. pyogenes* var. *aureus* Without serum	Dilution of lupulon	*M. tuberculosis* With 10% serum	*M. tuberculosis* Without serum
1:10 × 1000	+	—	1:10 × 1000	+	—
1:20 ,,	+	—	1:20 ,,	+	—
1:25 ,,	+	—	1:60 ,,	+	—
1:33 ,,	+	—	1:100 ,,	+	—
1:60 ,,	+	+	1:160 ,,	+	—
1:100 ,,	+	+	1:200 ,,	+	—
1:200 ,,	+	+	1:300 ,,	+	+
1:300 ,,	+	+	1:400 ,,	+	+
Control	+	+	Control	+	+

+ = growth; — = no growth.

[‡] The horse serum used in these experiments was kindly supplied by the Cutter Laboratories, Berkeley, Calif.

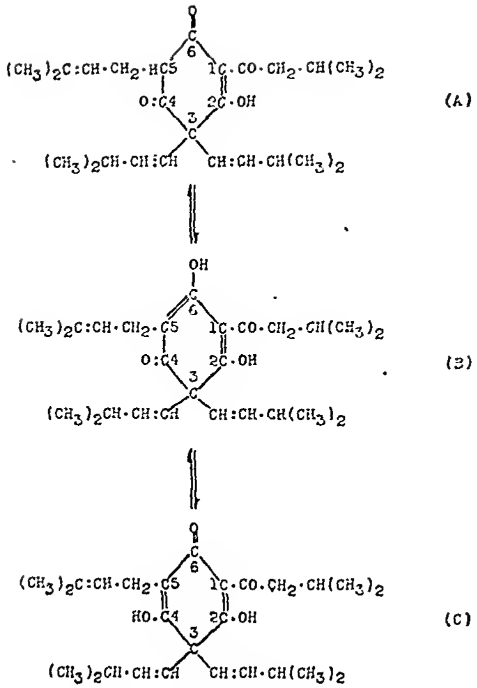

FIG. 1.

Lupulon may exist in any of the 3 forms shown above but probably (B) and (C) predominate.

methylene group in an extremely unstable arrangement. The double bonds in the 2 side chains in position 3 are susceptible to oxidation by air. The semi-aromatic system itself is, in general, an unstable configuration.

One would expect the hydroxyl in position 6 of tautomer (B) or the hydroxyl group in position 4 of tautomer (C) to acetylate under normal conditions. The hydroxyl in position 2 could be acetylated only with difficulty because of chelation with the carbonyl group on the one hand, and the hindrance due to the alkyl group in position 3 on the other.

Summary and Conclusions. Lupulon, an antibiotic extracted from hop resins, was found to be effective against Gram-positive bacteria *in vitro* but inactive against Gram-negative organisms. The antibiotic had no demonstrable effect on a number of pathogenic and nonpathogenic molds and actinomycetes.

Lupulon displayed no activity in the presence of horse serum. In the absence of serum the antibiotic exhibited its greatest activity against *M. tuberculosis* H37Rv. This may have been due to the fact that the tubercle bacillus was cultivated in a mineral medium with only an appreciable amount of interfering organic matter whereas the other organisms were grown in a medium containing peptone. The addition of serum to both media completely inactivated the antibiotic.

Lupulon is slowly inactivated in the presence of air and more rapidly in the presence of serum. Because of its inactivation by serum, the antibiotic is not suitable for clinical administration.

TABLE IV.
Effect of a Freshly Prepared and an Old Solution of Lupulon on *Mycobacterium tuberculosis* H37Rv.

Dilution of lupulon	Freshly prepared solution	Solution 10 days old
1:60 × 1000	—	—
1:80 "	—	—
1:100 "	—	+
1:140 "	—	+
1:180 "	—	+
1:200 "	—	+
1:300 "	—	+
Control	+	+

+ = growth; — = no growth.

16944

Effect of Autonomic Blocking Agents on Sweat Secretion in Cat.

HARRY D. PATTON. (Introduced by L. D. Carlson.)

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Sweat glands, although innervated by sympathetic postganglionic fibers, are excited by parasympathomimetic drugs (acetylcholine, mecholyl) and blocked by atropine. Dale and Feldberg[1] found that eserinized perfusates collected from the cat's foot during stimulation of the sacral sympathetic ganglia gave positive pharmacological tests for an acetylcholine-like substance. Perfusates collected during stimulation were inactive, if the sweat glands were excluded from the circulation by ligating the base of the footpad. The sudomotor system was thus shown to be anatomically sympathetic, but pharmacologically parasympathetic (cholinergic).

A recent study by Haimovici[2] suggests, in addition, an adrenergic component of sudomotor innervation. He points out that ephedrine, benzedrine and neosynephrine, all sympathomimetic drugs, evoke moderate sweating in man. Using a qualitative colorimetric method for detecting sweat secretion, he noted depression of both spontaneous and neosynephrine-induced sweating in man after injection of Dibenamine (N, N-dibenzyl-β-chlorethylamine hydrochloride), although mecholyl still induced profuse sweating. Since numerous studies indicate that Dibenamine[3] is a specific adrenolytic and sympatholytic drug, he concludes that suppression of sweating by Dibenamine indicates, in addition to the known cholinergic innervation, an "adrenergic component in the nervous mechanism of sweating in man."

In the present experiments the effects of Dibenamine and atropine on sweat secretions in the cat are studied by a quantitative procedure. Electrical stimulation of the lumbar sympathetic chain evokes transient electrical negativity in the skin of the footpad, relative to gland-free skin of thigh, back or ear.[4,5] The potential is the result of sweat gland activity; and its magnitude is a quantitative measure of the number of neuroglandular elements active. The response to a constant single shock to the sympathetic chain is relatively constant over long periods of time, permitting ready recognition of deviations due to injected drugs.

Methods and materials. In 5 cats, anesthetized with sodium pentobarbital, the lumbar sympathetic chain was exposed retroperitoneally. Single square wave shocks, 0.5 msec. in duration and of supramaximal intensity, were delivered to the L5-L6 interganglionic segment of the chain every minute for 5-15 minutes before, during, and for 30 minutes to one hour after drug infusion.

The potentials were recorded through Pb-$PbCl_2$ electrodes attached to the shaved skin of the thigh and to the ipsilateral hind footpad. The latter electrode, designed by pouring lead around a positive plaster impression of a cat's foot, established firm contact with all parts of the hairless foot and toe pads. The potentials were recorded without amplification on a string galvanometer or on a Hathaway galvanometer after amplification by a DC amplifier of the carrier type.*

The Dibenamine solution, prepared freshly in slightly acid saline, was administered through the femoral vein slowly (approximately 1 mg/min.) to avoid the complications of precipitous hypotension. Total dosage was recorded at each one-minute interval; final total dosages ranged from 15-20 mg per kilo of body weight. The pilomotor response of the tail to repetitive stimulation of the sympathetic chain was noted at the beginning and end of each experiment to assay qualitatively the effectiveness of the dosage.

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[1] Dale, H. H., and Feldberg, W., *J. Physiol.*, 1934, **82**, 121.

[2] Haimovici, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 40.

[3] Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

[4] Richter, C. P., and Whelan, F., *J. Neurophysiol.*, 1943, **6**, 191.

[5] Patton, H. D., *J. Neurophysiol.*, 1948, **11**, 217.

* The amplifier was designed and constructed by Mr. R. S. Bark, Department of Physiology and Biophysics, University of Washington School of Medicine.

Atropine sulfate was given in a similar fashion, except that the total dosage was administered rapidly.

Results. Slow intravenous infusion of Dibenamine in dosages (15 to 20 mg per kg) sufficient to block completely the pilomotor response of the tail, to produce miosis and to relax the nictitating membrane did not diminish the electrical response of sweat glands to preganglionic sympathetic stimulation. Fig. 1 shows the results of a typical experiment. At no time during the infusion did the magnitude of the potential drop below the control value. In some experiments longer post-infusion controls showed that the potentials were maintained at control heights as long as an hour after administration of the full dose. Indeed, in some animals the response increased moderately during and following Dibenamine dosage (Fig. 2). In only one experiment did the response show a significant decrease (35 minutes after full dosage); in this case it was found that the initially chosen stimulus strength was too weak, and the response was restored with increased strength of stimulus.

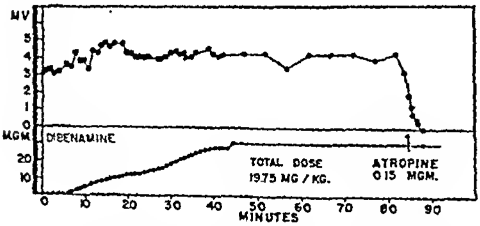

FIG. 1.
Upper graph, magnitude of footpad potential (m.v.) in response to supramaximal sympathetic stimulation. Lower graph, cumulative dose of Dibenamine. At the end of the experiment, injection of atropine rapidly abolished the responses.

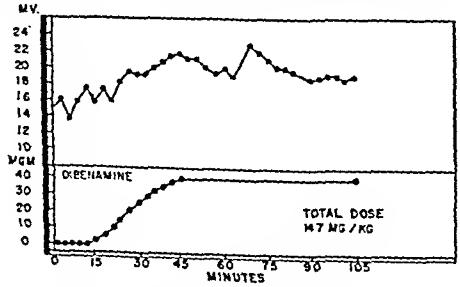

FIG. 2.
Increase in footpad potentials following Dibenamine administration.

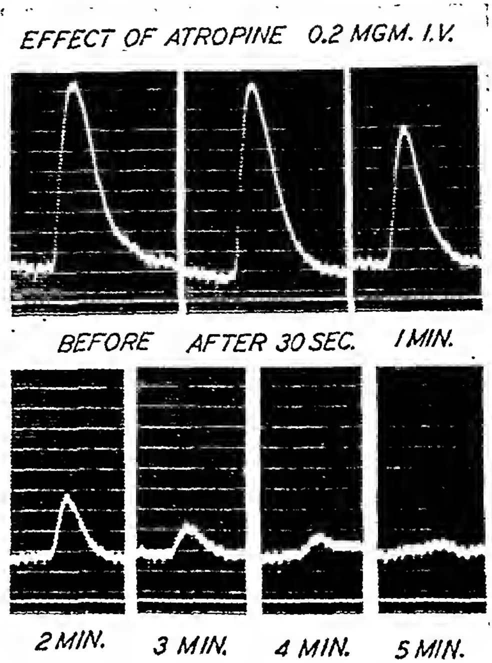

FIG. 3.
Potential responses of footpad to single shock stimulation of the sympathetic chain before and after injection of atropine.

Injection of atropine (0.1 mg per kg) produced a rapid and complete block of the sudomotor system. Fig. 3 shows the results of a typical experiment.

Discussion. The results indicate that sweat secretion in the cat is not blocked by Dibenamine, even with doses 3-4 times as great as that reported to block sweating in man. Miosis, relaxation of the nictitating membrane, and paralysis of the pilomotor system indicated that the dosage was adequate to block known adrenergic systems. In some animals the magnitude of the footpad potential actually increased during infusion of the drug.

This may have been due to vasodilation and increased blood supply to the glands.

On the other hand, irreversible sudomotor paralysis consistently followed injection of atropine. It is thus concluded, in agreement with Dale and Feldberg, that the sudomotor innervation in the cat is entirely cholinergic. Either there exists a marked species difference in cat and man in this respect, or the qualitative methods of observing sweating are misleading.

Summary. The effect of autonomic blocking agents on sweat gland response to single shock excitation of the sympathetic chain was studied in cats by a quantitative and objective method. Dibenamine, an adrenolytic and sympatholytic agent, failed to block sweat secretion whereas atropine consistently produced complete and irreversible sudomotor paralysis. It is concluded that the sudomotor innervation in cat is entirely cholinergic and possesses no adrenergic component, as reported in man.

16945

Stable, Reduced, Desiccated Streptolysin "O."*

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A technic has been described[1] for the determination of the antistreptolysin "O" titer of serum which involved the employment of a concentrated lysin, diluted and reduced to the active form with a solution of cysteine hydrochloride at the time of use. This method has proved to be very satisfactory. Large amounts of the lysin have been prepared not oftener than every 12 months and deterioration during storage at circa 2°C has rarely been observed. A stable material not requiring refrigeration nor reduction at the time of use would be most desirable. This report describes a procedure for the preparation of a desiccated, reduced streptolysin "O" which, upon reconstitution with distilled water, is immediately ready for use.

Streptolysin. Concentrated streptolysin "O", prepared exactly as described in the paper mentioned above, is used.[1] Complete removal of sulphate ions is determined after dialysis by the addition of a drop of 25 percent barium chloride solution to a small amount of the concentrated material. A precipitate forms after dialysis and the addition of sodium chloride. It should be removed by centrifugation.

Cysteine Hydrochloride. The dry salt is required.

Concentrated Buffer. A concentrated (4 times normal) buffered saline solution is prepared according to the following formula.

	g
NaCl	68.0
KH_2PO_4	45.2
$Na_2HPO_4 \cdot 12\ H_2O$	74.6
Distilled water to make 4 liters.	

After the salts have been dissolved the acidity is checked with a glass electrode pH meter and adjusted to final pH of 6.5. The solution need not be sterilized.

Buffered Solvent for Cysteine. Seven and two-tenths grams of solid NaOH is added to one liter of normal buffered saline (1 part of concentrated buffer saline diluted with 3 parts of distilled water). At the time of reduction and dehydration an M/5 solution of cysteine hydrochloride is prepared by dissolving 3.0 g of the dry salt in 100 cc of this alkaline buffer. Final pH should be 6.5 to 7.2. The solution need not be sterilized.

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* This investigation was conducted under the auspices of the Commission on Acute Respiratory Diseases, Army Epidemiological Board, Office of the Surgeon General, United States Army, Washington, D.C.

[1] Rantz, L. A., and Randall, E., PROC. SOC. EXP. BIOL. AND MED., 1945, 59, 22.

TABLE I.

	Volume of reagents			
	Lot 1		Lot 2	
	For 1 bottle, ml	For 100 bottles, ml	For 1 bottle, ml	For 100 bottles, ml
Vol. of concentrated lysin containing 40 combining units	.875	87.5	1.5	150.0
Vol. of M/5 cysteine	2.5	250.0	2.5	250.0
Vol. of normal buffer saline	(16.62)		(16.0)	
Vol. of conc. buffer saline	4.15	415.0	4.0	400.0
Total to be added to each bottle before lyophilization	7.52	7.52	8.0	8.0

Method. The object of the method is to have the lysin, salts and reducing agent combined in the proper proportions and in a volume conveniently small for lyophilization. Each finished bottle of dehydrated material contains exactly 40 combining units of streptolysin, the residue from 2.5 ml of M/5 cysteine solution and sufficient buffer salts so that the whole will be isotonic when reconstituted with 20 cc of distilled water. The following calculation is made: 20 ml less the volume of lysin containing 40 combining units plus 2.5 cc of M/5 cysteine equals the volume of normal buffer required. This value, divided by 4, gives the amount of concentrated buffer per bottle. The sum of the volumes of lysin containing 40 combining units, of M/5 cysteine, and of concentrated buffer saline is the amount of reduced buffered material to be transferred to each bottle before dehydration. The examples shown in Table I are illustrative.

The concentrated lysin, M/5 cysteine solution, and concentrated buffer are appropriately measured and mixed after complete preparations have been made for freezing and drying. The solution is dispensed with an accurate automatic pipette into standard 20 cc vials with an inside neck diameter of 12 mm. The maximum time which may elapse after reduction and before freezing is not known but 500 to 1,000 bottles may safely and satisfactorily be handled.

The filled bottles are vertically frozen at once in a shallow bath of methylcellosolve containing large amounts of dry ice, and desiccated from the frozen state in a commercial dehydrating apparatus at a pressure of 300 microns of mercury for 42 hours. The final moisture content is circa 2.5%. The vacuum is broken with sterile dry air and the bottles closed with a rubber flange stopper in a dry sterile stoppering cabinet and sealed with standard aluminum seals.†

The Test. One bottle of dehydrated, reduced lysin is reconstituted with 20 ml of distilled water. It dissolves at once to form a water clear solution and is ready for use. One combining unit is contained in 0.5 ml. The material may be used in any of the technics for determination of the antistreptolysin titer of serum. The dilution system previously described has proved to be very satisfactory.[1]

Stability. The dehydrated reduced lysin has been stored at 37°C, room temperature, and at 2°C for 18 months. Serial tests have been made and no loss of potency or alteration in the characteristics of the product has been noted. After reconstitution the material is highly unstable and should be used within 2 hours.

Discussion and Summary. A method is described for the preparation of a very stable, desiccated, reduced streptolysin "O". The material, when reconstituted with distilled water, is suitable for use in the determination of the antistreptolysin "O" titer of human serum. The availability of this material greatly simplifies this procedure since it is always immediately ready for use without the addition of the reducing agent. In addition, it may be shipped and stored conveniently since refrigeration is not required. Its production on a large scale or commercial basis would not prove difficult if a sufficient need for this material should arise.

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† The cooperation of the Cutter Laboratories is gratefully acknowledged. Processing has been done in the plant of this company. Mr. B. E. Emery of that organization may be consulted for additional technical details.

16946

Influence of Heating on the Liberation of Certain Amino Acids from Whole Soybeans.*

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The effect of heating on the nutritive value of soybeans has been studied by a number of investigators. A review of such studies was made recently by Evans and McGinnis.[1] They further showed that the nutritive value of soybean proteins as determined by gain in weight per gram of protein consumed by chicks was increased when the soybean oil meal was autoclaved at 100°C, 110°C or 120°C for 30 minutes but to a smaller degree when it was autoclaved at 130°C for 30 or 60 minutes. The changes in nutritive value were found to parallel the availability of the methionine and cystine of the soybean.

Clandinin *et al.*[2] reported that heating solvent-extracted soybean flakes in an autoclave at 15 lb pressure for 4 minutes resulted in a meal of high nutritive value for the chick; however, when the heating was prolonged to 4 hours a decrease in the nutritive value was observed. These changes were shown by Riesen *et al.*[3] to be related to an increase in the amount of each of the essential amino acids liberated by pancreatic hydrolysis with the short period of heating and a decrease with the prolonged heat treatment.

More recently Klose *et al.*[4] reported that treatment of raw soybeans with steam at 15 lb pressure gave a product of maximum nutritional value for the rat after 10 to 15 minutes while heating beyond this time resulted in a gradual decrease owing to a resultant deficiency of methionine, lysine and leucine. Steam treatment at about atmospheric pressure for 30 minutes gave a product showing a slightly higher maximum and there was little decrease in nutritive value on prolonged heating.

Soybeans have been used in the Chinese diet for centuries and one of the methods of cooking is to boil in water for 2 to 3 hours until the beans are quite soft. The present investigation was designed to determine the effect of boiling soybeans in water at different periods of time on the liberation of certain essential amino acids by acid and by enzymatic digestion.

Methods. The soybeans used were recently harvested, dried seeds of the Hawkeye

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† On leave from the Institute of Nutrition, National Institute of Health, China, and on traveling fellowship of the World Health Organization of the United Nations and of The Williams-Waterman Fund for the Combat of Dietary Diseases.

[1] Evans, R. J., and McGinnis, J., *J. Nutrition*, 1946, **31**, 449.

[2] Clandinin, D. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.*, 1947, **24**, 150.

[3] Riesen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W., *J. Biol. Chem.*, 1947, **167**, 143.

[4] Klose, A. A., *Hill, B., and Ferold, H. L., Food Tech.*, 1948, **2**, 201.

TABLE I.
Effect of Time of Boiling on the α-amino Nitrogen Liberation from Soybeans.

Time of digestion, hr / Time of boiling hr	α-amino nitrogen (g %)			
	Acid digest	Pancreatic digest		
	10	0	6	25
0	4.51	.42	0.68	1.00
¼	—	.44	0.99	1.48
1	—	.54	1.63	2.00
2	4.56	.49	1.57	2.12
3	4.75	.52	1.60	2.10
4	4.70	—	—	—
12	4.52	.49	1.51	2.01

variety.[‡] They were washed free from dirt wiped free from moisture and then air dried at room temperature. Weighed samples of whole beans were soaked in water for 3 hours and then boiled in water (about 5 times the weight of the beans), under reflux for varying periods of time. The soaked and the heated soybeans with liquid were then homogenized in a Waring blendor for about one-half hour at short intervals to prevent heating of the emulsion.

These emulsions were used for subsequent acid and alkaline hydrolysis by autoclaving at 15 lb with final volume at 20 ml of 3 N hydrochloric acid per 0.5 g soybean protein for 10 hours and 10 ml of 5 N sodium hydroxide per 0.5 g of soybean protein for 15 hours, respectively. Alkaline hydrolysis was carried out for the assay of tryptophan.

Enzymatic hydrolysis was carried out by shaking the emulsion with desiccated and defatted whole pancreas (VioBin 3 × USP Trypsin) in disodium phosphate buffer (pH 8.3) with toluene at 37°C for 0, 6 and 25 hours. The quantities used were 100 mg whole pancreas and 50 ml of 0.2 M Na_2HPO_4 to one gram of protein. Other details were similar to those described by Riesen *et al.*[3]

The liberation of α-amino nitrogen from the acid and pancreatic hydrolysates was determined by the nitrous acid micromethod of Van Slyke.

Amino acid assays were carried out according to the method of Henderson and Snell[5] except that in the present experiment *Leuconostoc mesenteroides* P60 was used for leucine and tryptophan while *Leuconostoc citrovorum* 8081 was used for arginine, cystine, histidine and methionine. Lilly's liver concentrate (Reticulogen 20 × USP) was added to the media for the latter organism at a level of 0.4 ml per 500 ml of medium (Steele *et al.*[6]).

Results and Discussion. Liberation of α-amino nitrogen. As shown in Table I the total α-amino nitrogen found in soybean after acid hydrolysis was unaffected by boiling for different periods of time up to 12 hours. With pancreatic digestion the amount of α-amino nitrogen liberated from raw soybean and that which had been boiled for 15 minutes was small but reached a maximum when the soybean was heated for one hour. Prolonged heating for periods of 2, 3 or 12 hours produced practically no difference in the total α-amino nitrogen liberated. These results are similar to those reported previously[3] for solvent-extracted flakes except that in the previous experiment heating at the higher temperature for 4 hours resulted in a decrease in α-amino nitrogen released by pancreatin. The fact that boiling for 15 minutes brought about a smaller release of α-amino nitrogen by pancreatin than boiling for longer periods is added evidence that the trypsin inhibitor was not the sole cause of the smaller release from raw soybean. This confirms the

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[‡] Kindly supplied by Dr. J. H. Torrie of the Agronomy Department, University of Wisconsin.

[5] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.

[6] Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.*, in press.

TABLE II.
Liberation of Amino Acids from Raw and Boiled Whole Soybeans.

	Amino acid N content of soybean (% of total N)[*]			% amino acids liberated by pancreatin (25 hr digestion)		
Boiling time, hr	0	3	12	0	3	12
α-amino nitrogen	67.3	71.0	67.5	22.2	44.2	44.5
Arginine	14.3	14.8	14.5	28.1	61.1	55.4
Cystine	0.50	0.58	0.57	30.2	76.6	60.6
Histidine	4.13	4.82	4.74	21.1	44.7	43.1
Leucine	4.41	4.22	3.99	30.0	45.7	50.7
Lysine	6.88	6.87	6.88	26.6	53.7	50.5
Methionine	0.60	0.62	0.63	33.5	72.3	63.9
Tryptophan	0.60	0.61	0.57	35.4	79.0	78.3
Total[†]	31.4	32.5	31.9	28.5	55.4	53.2

[*] Total nitrogen of soybean determined by Kjeldahl method was 6.69 g %.

[†] Amino acid N totals are sums of the percentage of the amino acids N liberated by acid or alkaline hydrolysis. Pancreatic hydrolysis totals are obtained by dividing the total amount of individual amino acids liberated enzymatically by the total liberated by acid or alkaline hydrolysis.

finding of Riesen *et al.*[3] and also Westfall and Hauge[7] although the latter workers believe that the trypsin inhibitor was the chief cause of poor utilization of the protein by the mouse.

Amino acid levels. The results of heating on the individual amino acid value of soybeans in the raw state and after boiling for 3 and 12 hours are shown in Table II. It will be noted that after acid hyrolysis the amino acid values for raw soybeans are essentially the same as those boiled for 3 or 12 hours. Riesen *et al.*[3] who used prolonged heating at 15 lb pressure found a decrease in the liberation of lysine, arginine and tryptophan. The difference may be due to the fact that the temperature in the experiment of Riesen *et al.* was much higher than that of the present experiment.

Recently Patton *et al.*[8] reported that boiling of soy globulin in 5% glucose with refluxing for 24 hours caused a considerable loss in 4 out of 10 essential amino acids determined, namely lysine, arginine, tryptophan and histidine, while boiling in water resulted in no change in these amino acids. Our results with whole soybean were similar to theirs with boiling in water and would indicate that the sugars and other carbohydrates in soybeans do not play a part in the loss of amino acids during boiling, although browning did occur in samples boiled longer than 3 hours. It appears that the loss of certain amino acids during boiling in the presence of glucose reported by Patton *et al.*[8] does not have a counterpart in the whole soybean moiety.

Block *et al.*[9] showed that baking of a cake mix of flour, sugar, egg white, lactalbumin, hydrogenated vegetable oil, dried yeast, molasses and salt with 25% of its calories as protein caused a decrease in the protein efficiency. The decrease they believe was possibly due to a partial destruction of lysine which occurred during the baking, or to a formation of a new peptide linkage by a reaction of the free carboxyl groups of the dicarboxylic amino acids with the α-amino groups of lysine, the new peptide linkage being resistant to enzymatic digestion but not to acid hydrolysis. Since during baking the temperature on the surface of the cake may be considerably higher than 100°C, a partial destruction of lysine may occur similarly to that shown by Riesen *et al.*[3] On the other hand the molasses may furnish a considerable amount of glucose to cause destruction of lysine as reported by Patton *et al.*[8]

Pancreatic digestion. The effect of boiling the soybean on the liberation of amino acids was quite evident after 25 hours of pancreatic digestion (Table II). The total *a*-amino nitrogen released from the sample boiled for 3 hours was double that of the raw sample. Prolonged boiling for 12 hours resulted in no further change. Similar release occurred with each of the 7 amino acids studied. Since previous work indicated that the 7 amino acids tested in this study might be susceptible to damage by heat and in view of the present findings no further study was made of the remaining essential amino acids.

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[7] Westfall, R. J., and Hauge, S. M., *J. Nutrition*, 1948, **35**, 379.

[8] Patton, A. R., Hill, E. G., and Foreman, E. M., *Science*, 1948, **108**, 659.

[9] Block, R. J., Cannon, P. R., Wissler, R. W., Steffee, C. H., Jr., Straube, R. L., Frazier, L. E., and Woolridge, R. L., *Arch. Biochem.*, 1946, **10**, 295.

The results of the present study do not support the view that methionine is released at a greater rate than leucine and lysine after heating (Melnick *et al.*[10]). It was shown here as well as in a previous report[3] that heat increases the release of all amino acids from soybean protein by pancreatin to a similar extent. It would appear that the sulfur amino acid deficiencies in raw soybean previously reported by Hayward *et al.*[11] are due to a limited content *per se* of these amino acids in the soybean protein. The low quantities of cystine, methionine and tryptophan in both raw and heated soybean are evident from Table II. Supplementation of the protein with cystine and/or methionine should increase the nutritional value of both raw and heated soybean. Furthermore since all amino acids are less available in raw soybean the critical nature of the sulfur amino acid deficiencies in this protein is even more striking than in the heated soybean.

It is of interest to point out that the percentage increase of total amino acids released by pancreatin over the percentage of *a*-amino nitrogen is considerably smaller than that reported previously.[3] This may be explained by the fact that the organisms used in the present experiment did not utilize peptides to such an extent as those organisms employed in earlier experiments.

The results of our study show that the method of cooking soybean used in China does increase the nutritive value of soybean by allowing a greater enzymatic release of essential amino acids and that there is no destruction on prolonged boiling up to 12 hours. In view of this and the fact that cooking at higher temperature (autoclaving at 15 lb) results in a deleterious effect on certain amino acids reported previously,[3] cooking of soybean in a pressure cooker should not be introduced until future studies have proven that under the ordinary cooking condition with a pressure cooker no harmful result is obtained.

Summary. A study was made on the effect of boiling on the liberation of amino acids by acid and enzymatic hydrolysis using microbiological assay methods.

Boiling up to 12 hours did not not affect the release by acid hydrolysis of *a*-amino nitrogen and of 7 essential amino acids studied.

Boiling for 15 minutes caused a slight increase in the release of *a*-amino nitrogen and amino acids by pancreatic digestion but boiling for 1 hour and longer up to 12 hours resulted in a marked increase. The increase in the enzymatic liberation of amino acids for periods of over one hour was about twice that liberated from raw soybean. Prolonged boiling up to 12 hours with definite browning did not result in a decrease in enzymatic liberation of amino acids from the "overcooked" soybean.

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[10] Melnick, D., Oser, B. L., and Weiss, S., *Science*, 1946, **103**, 326.

[11] Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219.

16947

Colorimetric Determination of Urethane as Ethyl Alcohol in Blood.*

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Archer, Chapman, Rhoden and Warren[1] have described a method for the determination of urethane (ethyl carbamate) in blood in connection with urethane therapy in leukemia. Their method was based upon the hydrolysis of urethane to ethyl alcohol and determination of the latter by oxidation with dichromate and titration of excess dichromate by iodimetry. This procedure, in specimens of normal blood, gave a relatively high nonspecific "urethane" value of 3-5 mg %; the clinical levels were 10-20 mg %. No recovery experiments were reported.

For the purposes of studying the blood urethane level in patients receiving urethane locally for therapy[2] against gram-negative organisms in infected wounds, a method was needed with greater precision at lowel levels of urethane. Hemingway, Bernat, and Maschmeyer[3] have indicated the unsatisfactory results of iodimetry in micro-titrations of chromic acid, and have proposed the use of barium diphenylamine sulfonate as an indicator, and ferrous sulfate as the titrant. For convenience we have preferred a colorimetric procedure; for increased accuracy we have introduced a more concentrated blood filtrate. The ferrous titration has been, in our hands, equally precise but more time-consuming.

Reagents. 0.300% potassium dichromate. 27 N sulfuric acid. 3 vol. of concentrated acid added to 1 vol. of water. 50% sodium hydroxide (wt.-vol.). 2/3 N sulfuric acid. 10% sodium tungstate.

1:5 tungstic acid blood filtrate. 2 volumes of water are added to oxalated blood, preferably at least 12 ml for blood low in urethane, and the mixture is shaken occasionally for a few minutes. One volume each of 10% sodium tungstate and 2/3 N sulfuric acid is added. It is mixed well and allowed to stand for 5 minutes, after which it is centrifuged and the supernatant fluid is filtered.

Distillation apparatus. An all-glass distillation apparatus is used, consisting of a glass-stoppered 125 ml pyrex distilling flask with an air-cooled condenser ¼ inch in diameter and 15 inches long. This leads to a vertical delivery tube 7 inches long ending in an aeration bulb. The aeration bulb dips below the surface of the acid-dichromate solution contained in a 25 ml pyrex glass-stoppered graduated cylinder cooled in an ice water bath in a liter beaker. The ice bath rests on a wooden block high enough (about 3½ inches) so that the aeration bulb will be well above the 13 ml mark on the cylinder when the block is removed and the ice bath with the cylinder is lowered to the table.

Blank determination. 16 ml of tungstic acid filtrate and roughly 4 ml of water or less filtrate and enough water to make a 20 ml volume are added to the distilling flask containing a few glass beads. 1 ml of 0.300% potassium dichromate and 3 ml of 27 N sulfuric acid are added to a 25 ml glass-stoppered cylinder and mixed. The cylinder is placed in the ice bath with the ice water covering the 16 ml mark. The delivery tube is lowered into the cylinder so that the aeration bulb is near its bottom. The distillation is carried out with a micro-burner and must be fast enough to prevent the acid-dichromate solution from sucking back. About 8.5 ml are distilled. To stop the distillation, the ice bath with the cylinder is lowered to the table while the flask is still being heated. The outside of the delivery tube is washed with a few tenths ml of water, which is blown

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* Aided by a grant of the Committee on Therapeutic Research of the Council of Pharmacy and Chemistry of the American Medical Association.

[1] Archer, H. E., Chapman, L., Rhoden, E., and Warren, F. L., *Biochem. J.*, 1948, **42**, 58.

[2] Howe, C. W., *Surg., Gynec., and Obstet.*, 1948, **87**, 425.

[3] Hemingway, A., Bernat, L. A., and Maschmeyer, J., *J. Lab. and Clin. Med.*, 1948, **33**, 126.

into the cylinder by heating the flask. The volume in the cylinder is made to about 12.9 ml with additional distillate (or water).

The cylinder is stoppered, shaken, sealed with 27 N sulfuric acid, and heated at 85° for 60 minutes. It is cooled in water, made up to a volume of 13 ml and mixed. The contents are transferred to an Evelyn tube and read in the Evelyn colorimeter using a 440 mμ filter[4] after the instrument has been set to 100% transmittance with water. The preparation of a standard curve is described below. A similar standardization should be done by each user of the method.

Urethane plus blank determination. The 25 ml cylinder containing freshly prepared acid-dichromate solution is set up with the distillation apparatus as in the blank determination. Sixteen ml of tungstic acid filtrate or less filtrate and enough water roughly measured to make a 16 ml volume are added to the distilling flask. 4 ml of 50% sodium hydroxide are added, the flask is stoppered and the contents are mixed. A water bath is set up around the distilling flask and heated at boiling for 15 minutes. To stop this heating, first the flame under the boiling water bath is increased until the acid-dichromate solution is forced out of the delivery tube. Then the ice bath is lowered to the table, the boiling water bath is removed, the ice bath is replaced and the distillation is started immediately. The rest of the procedure is as in the blank determination. Since the dichromate color is very stable, the determination may be interrupted either before or after the 85° incubation by storage in the refrigerator. The urethane concentration is obtained by subtracting the concentration of the blank from that of the urethane plus blank.

Urethane standard curve and formula. Different amounts of urethane, varying from 0.15 to 1.3 mg, were analyzed by the above method. Each quantity was determined 4 to 10 times. The average values of the optical densities were plotted against mg urethane (Fig. 1). The relationship was linear in this range. The value of the optical density with no urethane was taken as that of a determination with 16 ml water plus 4 ml of 50% sodium hydroxide. This value, 0.4297 ± 0.0068, was slightly lower than that, 0.4436 ± 0.0056, of the acid-dichromate solution simply diluted to 13 ml. The slope of the line was taken as the average of the separate slopes, omitting that of the 1.3 mg urethane point. Amounts of urethane greater than 1.2 mg showed a consistent deviation from this line. The formula of this line is:

Mg Urethane = 3.47 (0.4297 — L).

where the absorbency of the sample, or "optical density", L = log 100 — log G, and G is the Evelyn galvanometer reading (percentage transmittancy).

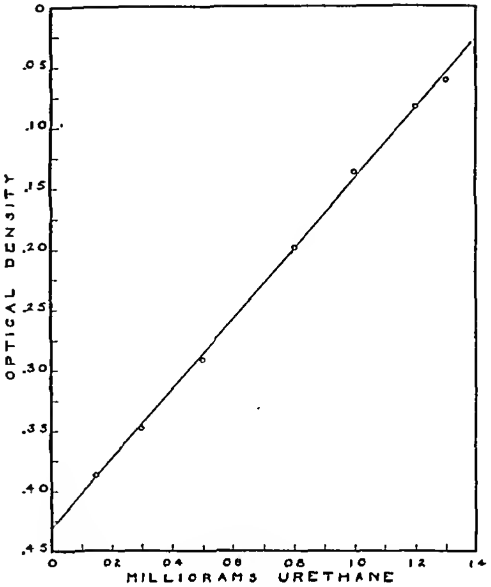

FIG. 1.
Urethane standard curve.

Precision of method. The precision of the above urethane standard determinations is given in Table I. Since the error of a single determination is within ± 2 standard errors, at 0.15 mg urethane the error is about 10% and this decreases to about 2% with 0.5 to 1.2 urethane.

Recovery of urethane added to blood. The recoveries of urethane added to whole blood at concentrations from 3.9 to 43.5 mg % using a 1:5 tungstic acid filtrate are recorded in Table II. These vary from 99 to 111%.

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[4] Gibson, J. G., and Blotner, H., *J. Biol. Chem.*, 1938. **126**, 551.

TABLE I.
Precision of Urethane Standard Determinations.

Urethane, mg	0.15	0.3	0.5	0.8	1.0	1.2
Standard error, in %	5.1	2.4	0.7	1.2	0.5	0.7

TABLE II.
Recovery of Urethane Added to Blood.

Urethane added, mg %	Urethane found, mg %	% recovery
3.9	4.2	108
5.7	6.3	111
7.8	7.7	99
11.1	12.3	111
15.9	16.1	101
18.5	19.3	104
19.2	19.0	99
32.3	34.5	107
43.5	45.7	105

Such bloods gave the same urethane values when they were previously hemolyzed with one tenth volume of 1% saponin. The 1:10 tungstic acid filtrate was abandoned because the recoveries were more variable at low urethane concentrations.

Specificity of method. In 5 normal bloods we have found non-specific "urethane" values varying from 0.1 to 1.4 mg % and averaging 0.8 mg %. Five other bloods from patients receiving drugs (sulfadiazine, streptomycin, penicillin, phenobarbital and amytal) in therapeutic doses gave "urethane" values from 0 to 1.1 mg % and averaging 0.5 mg %. These "urethane" values are zero within the accuracy of the method, hence urethane values below 1.5 mg % have no significance.

Clinical blood urethane levels. Patients with wound infections receive urethane locally.[2] In 2 such cases, we have found the blood urethane to be 3.1 and 5.6 mg %, after 4 and 8 days of treatment, respectively.

Application of method for ethyl alcohol determination. The blank determination in the urethane method may be used for the determination of ethyl alcohol since the conditions of the distillation and incubation were chosen to give quantitative recoveries of ethyl alcohol in amounts yielded in the urethane determination, that is, about ½ the amount of urethane. A standard alcohol curve was constructed from dilutions of a standard alcohol solution prepared from a weighed quantity of absolute ethyl alcohol. The curve was linear up to 0.4 mg alcohol, the 0.6 mg point deviating about 5 percent from the line. The equation of the line up to the 0.4 mg point was:

$$\text{Mg Ethyl Alcohol} = 1.65\,(0.4436 - L).$$

Strictly speaking the optical density at zero alcohol (0.4436 in the equation) should be that of a blank distillation with water.

Summary. A colorimetric method is described for the determination of urethane in blood, which gives 99 to 111% recoveries in the range 4 to 44 mg % urethane, and a non-specific "urethane" value of normal blood of 0.1-1.4 mg %.

A part of the method may be directly used for the determination of ethyl alcohol.

16948

Agglutination of Sea Urchin Eggs and Sperm by Basic Proteins.

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It is well known that basic proteins (protamines and histones) react with a wide variety of unrelated substances forming precipitates (with proteins, nucleic acids, etc.), causing cell agglutination (*i.e.* erythrocytes,[1] bacteria[1]) and exerting various physiological effects (*i.e.* parthenogenesis,[2] bacteriostasis[3]).

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[1] Lajmanovich, S., and Mittelman, N., *Rev. Inst. Bact.*, "Carlos G. Malbran," 1944, **12**, 320.

It is not surprising, therefore, that such substances precipitate the jelly coat of sea urchin eggs, agglutinate sea urchin eggs, and agglutinate the spermatozoa of a variety of forms.[4,5] It is unfortunate, however, that another sea urchin egg jelly precipitin and egg agglutinin, namely antifertilizin, should be confused with basic proteins from the sperm nucleus.

In a recent study Hultin[6] attempts to show that the sea urchin egg jelly precipitins (egg agglutinins) extracted from sea urchin sperm by Frank[7] (by heating sperm) and Tyler[8,9] (by freeze-thawing or extraction at pH3) are basic proteins, that they are non-specific in their action and that they are obtained from the sperm nucleus. Hultin maintains that the extracts of Frank and Tyler do not contain the sperm surface substance, antifertilizin, that reacts with the specific sperm agglutinin, fertilizin, obtained from eggs. The last is a particularly important charge since much of our present knowledge of the role of specific substances in fertilization stems from studies on sperm extracts as prepared by Frank and Tyler.

Hultin's claims are based on the following observations: (1) basic proteins obtained by extracting sperm with acid (pH 0.9 to 1.0) agglutinated eggs of various echinoid species indiscriminately, (2) basic proteins split from sperm nucleoproteins (cytoplasm free) caused egg agglutination and jelly precipitation, (3) sperm cytoplasm prepared by citric acid extraction had no effect on eggs or egg jelly of the homologous species (*Arbacia lixula*) whereas acid extracts of the residual sperm nuclei gave strong positive reactions with eggs, (4) egg agglutinin and jelly precipitin samples prepared from sea urchin sperm by heating or extraction with weak acid (pH 3) gave positive tests for desoxyribosenucleic acid. Hultin makes no statement regarding the presence or absence of free basic protein in these last preparations.

Unfortunately Hultin reports no test for cross agglutination of sperm by heterologous fertilizin or cross agglutination of eggs by the sperm extracts of Frank and Tyler. Without such data as a standard of specificity his studies on specificities of sperm extracts have little significance since cross reactions between echinoid sperm and fertilizin are known. Hultin mentions no controls testing the effect of his basic protein extraction procedure (extraction at pH 1) or the effects of the cytoplasm extraction procedure (citric acid extraction) on antifertilizin samples as prepared by Frank and Tyler. To claim identity of the egg agglutinins of Frank and Tyler with the basic protein fraction it would appear essential to show that the former retains its activity when subjected to the extraction procedure required for preparation of the basic protein.

Some years ago the writer[4] began a study of the effects of basic proteins on eggs and sperm. It was found that basic protein prepared by acid extraction (pH 0.5 to 1.0) of sperm of 3 sea urchins (*Strongylocentrotus purpuratus*, *Lytechinus anemesis*, *Arbacia punctulata*) not only agglutinated homologous eggs, but agglutinated homologous sperm as well. The latter property is definitely not shared with sea urchin antifertilizin as prepared by Frank and Tyler. Such basic protein preparations also agglutinated sperm from a variety of unrelated species. Thus *Arbacia* basic protein agglutinated sperm of seven (three molluscs, four echinoderms) out of ten forms; *S. purpuratus* basic protein agglutinated sperm of 5 echinoderms and one mollusc. These and a variety of other basic protein sperm agglutinations will be reported in detail elsewhere.

Basic proteins give strong non-specific egg jelly precipitation and egg agglutination reactions as Hultin states. Thus basic protein

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[2] Loeb, J., Artificial Parthenogenesis and Fertilization, Univ. Chicago Press, 1913.

[3] Negroni, P., and Fischer, I., *Rev. Soc. Argentina Biol.*, 1944, **20**, 487.

[4] Metz, C. B., Doctorate Thesis, California Institute of Technology, 1942.

[5] Hultin, T., *Ark. Kemi, Mineral., Geol.*, 1947, **24B**, 12.

[6] Hultin, T., *Pubbl. staz. zool. Napoli*, 1947, **21**, 2.

[7] Frank, J. A., *Biol. Bull.*, 1939, **76**, 190.

[8] Tyler, A., *Proc. Natl. Acad. Sci. U. S.*, 1939, **25**, 317.

[9] Tyler, A., and O'Melveny, K., *Biol. Bull.*, 1941, **81**, 364.

preparations from sperm of 7 forms (one annelid, 3 molluscs, 3 echinoderms) all agglutinated *Arbacia eggs*. It is of interest also that seminal fluid from the 3 molluscs agglutinated the *Arbacia* eggs. However, the basic protein egg agglutinins prepared from sea urchin sperm appear to be separate and distinct from the egg agglutinin (antifertilizin) prepared by freeze-thawing sperm. This follows from the fact that the egg agglutinating property of such antifertilizin was reduced (*S. purpuratus*) or disappeared entirely (*Arbacia punctulata*) when the antifertilizin preparations were subjected to the procedure used to extract basic protein. Thus, a sample of *Arbacia* antifertilizin (titer 16), prepared by freeze-thawing sperm, was adjusted to pH 0.8. A precipitate which formed at pH 2 to 3 was centrifuged off 4 hours later and the supernatant was neutralized. The sample was diluted to only one half the original concentration by addition of acid and base. It had no effect on the jelly coat of *Arbacia* eggs. It appears then that the antifertilizin was either inactivated by the acid or that it separated out in the precipitate (not tested further). Extracts prepared from whole sperm by this method gave powerful egg and sperm agglutination. Further evidence that the egg agglutinins in sperm extracts prepared by pH 1 extraction (basic proteins) and by freeze-thawing (antifertilizin) are separate and distinct was obtained in a neutralization experiment. A pH 1 sperm (*S. purpuratus*) extract which agglutinated both eggs and sperm was mixed with an active antifertilizin extract prepared by freeze-thawing sperm. The resulting mixture had no effect upon either eggs or sperm. Apparently the egg agglutinins in the two extracts can not coexist.

One may conclude from these experiments that at least 2 distinct jelly precipitating and egg agglutinating agents can be extracted from sea urchin sperm. The egg agglutinin(s) in pH 1 sperm extracts is probably a basic protein which acts in a highly non-specific fashion. The freeze-thaw and probably also the heat and pH 3 sperm extracts contain a different egg agglutinin(s), namely antifertilizin. For a discussion of antifertilizin specificity the reader is referred to Tyler's[10] recent review. The electrophoretic studies of Runnstrom, Tiselius and Vasseur[11] and Tyler[10] indicate that antifertilizin preparations contain a single acidic protein. In view of the foregoing the writer believes that Hultin has confused these two egg agglutinating substances obtainable from sperm. In the cases where basic protein extracts agglutinate homologous sperm as well as homologous eggs, this confusion can be rather readily avoided since antifertilizin preparations act on eggs but not on homologous sperm.

Summary. The agglutination of sea urchin eggs and sperm by basic protein extracts of homologous and unrelated sperm (reported by Hultin,[5,6]) is confirmed. Experiments are cited which show that antifertilizin, another egg agglutinin obtainable from sperm, is not present in or identical with the egg agglutinating basic protein fraction of sea urchin sperm as Hultin claims. Thus two distinct egg agglutinins are obtainable from whole sperm. One of these, antifertilizin, is obtained by heating, freeze-thawing or pH 3 extraction (Frank,[7] Tyler,[8,10]); the other, a basic protein(s), is obtained by pH 1 extraction of sperm.

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[10] Tyler, A., *Phys. Rev.*, 1948, **28**, 180.

[11] Runnstrom, J., Tiselius, A., and Vasseur, E., *Ark. Kemi, Mineral., Geol.*, 1942, **15A**, 16.

16949

Effect of Glycine Upon Action of Insulin in Rabbits.

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It was found by Pollak[1] that a pronounced hyperglycemia is produced in rabbits by the subcutaneous injection of a 5% solution of glycine. One gram of glycine was found to raise the blood sugar level of an animal weighing 1700 g from 90 to 160 mg % within 2 hours. Izumi[2] and Schenk[3] made further studies of the effect of amino acids upon the blood sugar level of rabbits. Izumi found that glycine administered intravenously (1.7 g per kg body weight) caused but a slight rise in the level of blood sugar, producing no subsequent lowering below the normal level, and that glycine administered perorally (2.675 g per kg) caused a slight lowering in the course of a 4 hour period. He pronounced the general effect of administering protein hydrolysates to be that of mobilizing blood glucose either from glycogen stores or by its formation from amino acids. Schenk[3] made a comprehensive study of the effect which the peroral administration of relatively large amounts of amino acids and of mixtures of these has upon the blood sugar level. Of those amino acids which were found to lower this level the effect of glycine was the most pronounced. In determining the amount of glycine to be administered the weight of the animal was not taken into account. The response was, however, interpreted in accordance with the amount administered. One gram was found to produce a *hyper*glycemia while the generally administered dose of 1.5 g was found to produce a *hypo*glycemia of varied degrees of intensity in different animals. Thus, the hypoglycemia produced by 1.5 g in an animal weighing 2810 g was more pronounced than that in one weighing but 1260 g. The hypoglycemic state disappeared within 8 hours after the administration of the glycine. Self-administered doses were found to produce a similar hypoglycemia in man.

When relatively small amounts of insulin were injected subcutaneously immediately after the feeding of glycine the decrease in the level of blood sugar was found to be the summation of that produced independently by the insulin and by the glycine.

It was found in this laboratory, using rabbits which had been carefully standardized with respect to their insulin tolerance, that small amounts of glycine (3 mg per kg) which were injected simultaneously with insulin, either subcutaneously or intravenously, did not significantly affect the insulin tolerance of these animals immediately. When the same rabbits were retested some weeks later, however, the effect which the administration of glycine had produced was found to be so pronounced as to cause these animals to reach the convulsive state when they were injected with but one-half of the originally determined convulsive dose of insulin and to promote a corresponding decrease in the level of the blood sugar. Thus, three rabbits which had received from 3 to 5 intravenous injections of insulin and glycine on alternate days late in February did not exhibit any significant response due to the glycine at that time. The same rabbits, upon receiving one or two subcutaneous injections of a mixture of glycine and insulin late in May, reached a convulsive state when the amount of insulin administered was but one-half that of the originally determined convulsive dose, (0.6, 0.7, and 0.7 units per kg respectively). These amounts constituting but one-half of the original convulsive dose are smaller than any which had heretofore been found to be required in bringing any normal rabbit to a convulsive state. Two other rabbits, upon receiving subcutaneous injections of glycine

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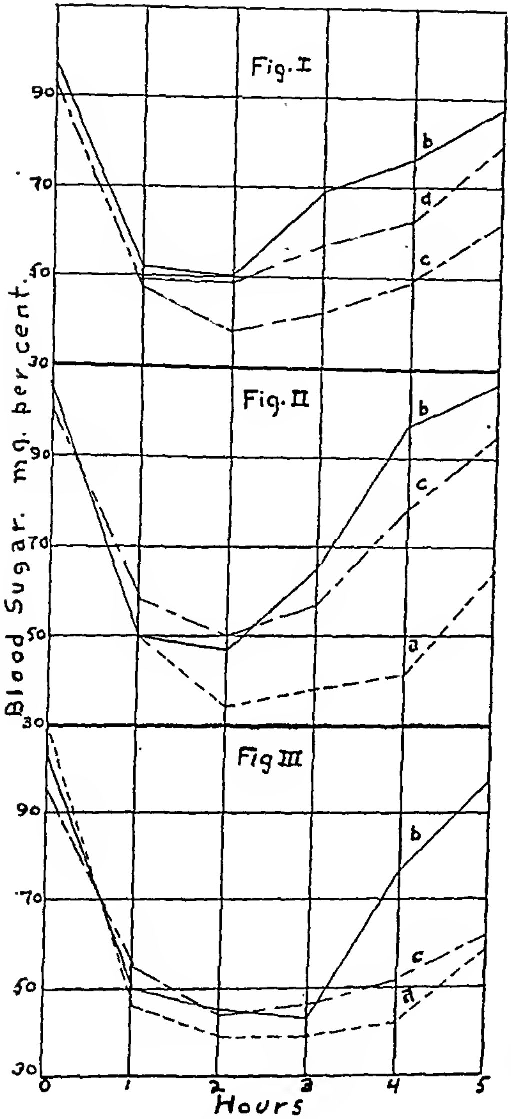
[1] Pollak, L., *Biochem. Z.*, 1922, **127**, 120.

[2] Izumi, K., *Biochem. Z.*, 1932, **248**, 383.

[3] Schenk, E. G., *Arch. Exp. Pathol. and Pharmacol.*, 1932, **167**, 201.



FIGS. 1-3.

Curve *a* of Figs. 2 and 3 shows the effect of a subconvulsive dose of insulin upon the blood sugar level of rabbits; curve *b* of each figure that of one-half the convulsive dose of insulin *before* treatment with glycine; curve c of each figure that of one-half of the convulsive dose of insulin *after* treatment with glycine; curve *d* of Fig. 1 that of one-half of a convulsive dose of insulin at a later period after treatment with glycine.

and insulin late in March, did not exhibit any significantly abnormal insulin response due to the glycine at that time. When the same rabbits received subcutaneous injections of insulin, with and without glycine, late in May, they were found to reach a convulsive state upon receiving but one-half of the originally determined convulsive dose of insulin.

This effect of glycine upon insulin action is more fully demonstrated by the following more recent data which were obtained in more carefully controlled experiments. These data, which are illustrated by the curves of Fig. 1 to 3, indicate the progressive change in the blood sugar level of rabbits for a 5 hour period following the subcutaneous injection of insulin, of glycine, and of a mixture of these. In all instances where glycine was administered the dose consisted of 3 mg per kg body weight. Five per cent solutions of glycine were used.

The data represented in Fig. 1 were obtained with a group of 4 rabbits (male albinos) which, as in all other experiments, had been carefully standardized with respect to their insulin tolerance. The data of curve *b* were obtained on Nov. 10th after the injection of one-half of the convulsive dose of insulin. Each animal was then injected with a mixture consisting of one-half of its convulsive dose of insulin and the usual amount of glycine on each of the following dates: Nov. 12th, 15th, 18th, 20th. The average blood sugar data obtained on each of these dates showed no significant deviation from those indicated by curve *b*. On Nov. 27th and 30th each animal again received the same injection of insulin and glycine as on Nov. 12th through 20th. The average data of these 2 days are given by curve *c*. They indicate a pronounced lowering of the blood sugar level during this 5 hour period as a consequence of the previous treatment with glycine. On Jan. 17th these rabbits were again injected with the same dose as on Nov. 27th. The data obtained, which are those of curve *d*, indicate a sustained effect due to the earlier injections of glycine.

The data of Fig. 2 were obtained with a second group of 4 rabbits which received similar treatment to those represented by Fig. 1. Injections of a mixture of glycine and one-half of the convulsive dose of insulin were given subcutaneously on Jan. 4th, 6th, 8th, and 10th. A similar injection was given each animal on Jan. 18th. Two of these (male albinos) reached the convulsive state after receiving the injection of Jan. 18th and their data are not included among those of Fig. 2. The other 2 rabbits (male grey) appeared to be somewhat less susceptible to the treatment than were the 2 albinos. Their data are represented by Fig. 2. The data of curve *a* were obtained on Dec. 20th following the injection of a dose of insulin slightly less than the convulsive dose and those of curve *b* on Dec. 22nd following the injection of one-half of the convulsive dose of insulin. Those of curve *c* were obtained on Jan. 18th following the injection of glycine and one-half of the convulsive dose of insulin. A definite effect of glycine on insulin action is also indicated here.

The data of Fig. 3 were obtained with a third group of 2 rabbits (black, male). After determining the response to a subconvulsive dose of insulin (curve *a*) and to one-half of a convulsive dose (curve *b*) these animals were injected with glycine only on Jan. 6th, 7th, 8th, 10th, and 12th. On Jan. 18th they received one-half of the convulsive dose of insulin but no glycine. A comparison of curve *c* with curves *a* and *b* indicates the effectiveness of the initial treatment with glycine upon the subsequent action of the injected insulin.

Many variables suggest themselves for further investigation. In view of the concern over the prediction of a possible shortage of insulin in the near future these experimental facts would seem of interest to a further search for a more favorable insulin economy. It should also be of interest to note how a similar treatment might affect humans who are predisposed to the conditions of diabetes.

Summary. Repeated injections of small amounts of glycine (3 mg per kg body weight) appear to increase the action of subsequent injections of insulin for a prolonged period of time.

The writer wishes to thank the donors of the Walter C. Hadley Fund for the generous aid which helped make this investigation possible.

16950

Production of Irritative and Destructive Changes in the Gastric Mucosa Followed by Regeneration.*

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Some progress has been made in the last 3 decades concerning our knowledge of the normal and pathological physiology of the stomach. Special emphasis has been lent study of the functions of the peptic and parietal cells; by contrast, our knowledge of the gastric mucous epithelium and its function is meagre. Hollander and his associates have been probing with care for some time this aspect of the problem of functions and morphology of the gastric mucosa; with few exceptions the functions of the gastric mucous epithelium and the other mucous secreting cells have not been given due consideration.

In this study, investigations of some functions of the mucous secreting cells were carried out; also the mechanism of regeneration of the gastric mucosa was studied.

Methods and experiments. The gastric mucous secreting cells were stimulated by placing various substances into the stomach with both ends ligated. Cats and dogs were employed in the experiment. Mustard oil and clove oil in concentrations of from 0.5 to 2 % (in corn oil), and eugenol in watery solutions up to 10% were used.

The animals were anesthetized with nembutal in doses of 15 mg per pound. When the operation was completed the animals were allowed to recover. After periods varying from 3 to 10 hours following operation, the animals were sacrificed.

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In another small group, after the period of exposure to the irritant, the abdomens of the animals were re-opened, the ligatures on the stomach were removed, the stomach was emptied and the wound was closed. The same procedure was repeated 2 or 3 weeks later. In all cases biopsies were taken before and after the completion of the experiment. In the group of surviving animals, biopsies were also taken 24 to 48 hours after the stimulation was stopped.

The specimens were immediately fixed with formalin and stained with hematoxylin-eosin and Mayer's muci-carmin.

Comparative studies of the action of the irritants on closed loops of small bowel and colon were also made. In these cases the procedure was essentially the same as that described by Florey and Webb.[1]

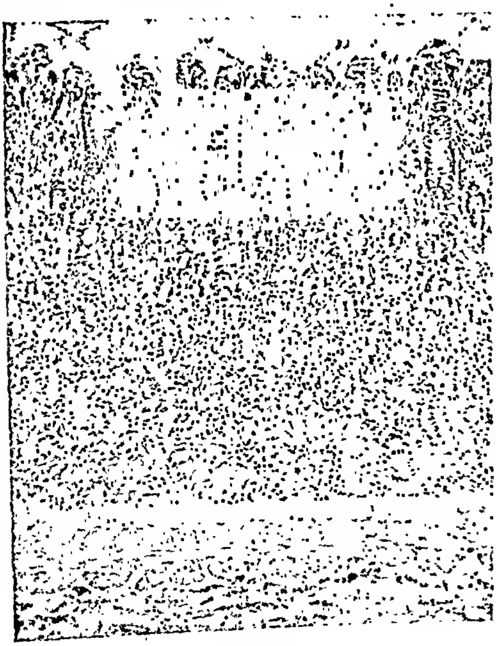

FIG. 1.

Stomach of a cat after 7 hours exposure to 1½% solution of mustard oil in corn oil. Early stage of mucosal changes; deepening of the foveolae, early epithelial desquamation. (Mayer's muci-carmin stain in all the microphotographs.)

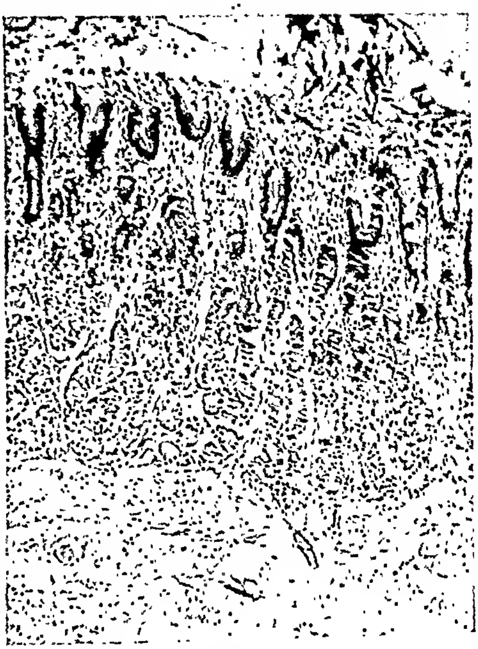

FIG. 2.

Stomach of a dog after 6 hours exposure to 10% aqueous solution of eugenol. This shows a more advanced stage than is shown in Fig. 1. There is definite loss of epithelium in the inter-foveolar areas.

Result. The results obtained in the colon and small bowel by Florey and Webb[1] were confirmed. In short, depletion of the goblet cells of the colon was readily produced. Similarly, diminution of the number of goblet cells was obtained in the small bowel.

In the stomach profuse macroscopic secretion of mucus was obtained with no obvious histological change. When the stimulation was stronger or the period of application increased, necrosis of the mucosa occurred. The process of necrosis was found to develop in the same general manner in colon, small bowel and stomach, except for the fact that the small bowel was the most susceptible and the stomach quite resistant to the irritative action. Edema, hyperemia, extravasation of red blood cells and leucocytic infiltration were noticed first. Later on, the most superficial portions

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[1] Florey, H., and Webb, R. A., *Brit. J. Exp. Path.*, 1931, 12, 286.

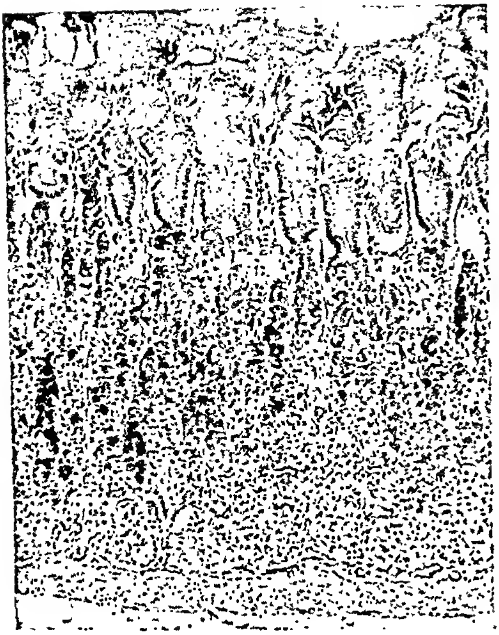

FIG. 3.
Stomach of a cat after exposure to 10% eugenol solution for 12 hours. Careful fixation of the whole stomach and attached mucus. The section shows a layer of epithelium already shed sticking to the new one.

of the epithelium were destroyed; the exposed cells of the stroma disintegrated and became spread over the denuded surface. A section, at that stage, showed an "umbrella" formed by cellular debris and mucous covering the mucosa. This layer acted as a protective covering and prevented necrosis of the walls of the pits. Further action of the irritant caused a progressive destruction of the remaining mucosa until ulceration occurred.

In the stomach, necrosis and regeneration were more thoroughly studied when irritants were applied to the mucosa. Deepening of the foveolae was first observed. (Fig. 1) Some time later the most superficial segments of epithelium became detached from the body of the mucosa, leaving only the deepest portions of the foveolae. (Fig. 2) If irritation ceased at this stage immediate regeneration took place. Under a protective layer of cellular debris and mucus, (Fig. 3) similar to the crust forming after injury to the epidermis), proliferation started at the bottom of the gastric pits. Growth of the cells from these areas restored the continuity of the surface epithelium and the mucosa looked thinner thereafter. The healed erosions in human stomachs show similar mucosal thinning. The process may be compared with the regeneration of the epithelium around skin appendages and the tips of the dermal papillae. If the entire thickness is destroyed, regeneration takes place from the margins of the wound. Similarly, if the entire thickness of the gastric mucosa is destroyed, regeneration takes place from the margins of the wound. Ferguson[2] demonstrated this experimentally and it can be seen also in pathological human material.

If destruction had not reached the bottom of the pits, it was followed by complete regeneration of the epithelial continuity in less than 48 hours. (Fig. 4)

In the cases in which a period of acute irritation was followed by recuperation of the animal, the procedure was repeated subse-

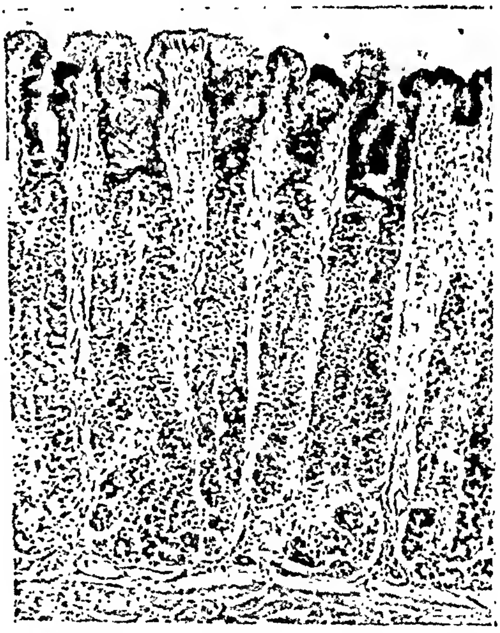

FIG. 4.
Same stomach as in Fig. 2, after regeneration. This biopsy was taken 40 hours after the stimulation was discontinued. The dark stained cells reaching almost to the muscularis mucosa are mucus-neck cells.

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[2] Ferguson, A. N., *Am. J. Anat.*, 1928, 42, 403.

quently. Biopsies of the mucosa before this second period of irritation was started showed changes consisting of cystic areas scattered in the body of the mucosa, areas of degeneration and a ragged contour of the epithelial surface as observed in the section; moreover, some foveolae were deeper than others, instead of exhibiting as is usual a fairly even depth. The mucosa, at this stage, proved to be more susceptible to the action of the irritant than in the initial period.

Discussion. The regeneration of the gastric mucosa as described may explain, in a very simple way, the much discussed resistance of this viscus to auto-digestion. We know that mucous secretion affords a protective mechanism to the gastric mucosa. The surface mucus restrains the acid-peptic juice from coming into direct and intimate contact with the mucosa. Evidence of this is offered by the experiments described, and has been obtained among others by Whitlow.[3] It is also known that mucus is increased whenever any kind of trauma, either mechanical or chemical, is applied to the mucosa. If, in addition to the layer of surface mucus, one considers the mucus contained within the epithelial cells, the concept of a more effective barrier suggests itself. Cells and mucus, the latter tenaciously adherent to the former, offer a protection which can be favorably compared to that present in the other segments of the gastro-intestinal tract. Moreover, before the cells of the epithelium loose their ability to secrete mucus they are shed and are immediately replaced by a layer of new ones, with a full secretory capacity. (Fig. 4)

If the rate of dissolution of the mucosa exceeds the rate of regeneration which may be variable, mucosal defects form, from which ulcers of the wall may develop. In the main, the regenerative capacity of the gastric mucosa is great and this condition accounts reasonably for the circumstance that the gastric mucosa does not digest itself.

Dragstedt and Vaughn[4] observed that spleen and kidney transplanted to the stomach were covered by a layer of columnar epithelium. Similarly, Varco and Wangensteen[5] noticed that omentum employed to cover stomach defects (in which approximation of the gastric walls was precluded by the insertion of metallic rings), became covered by gastric epithelium. Protection against digestion in these tissues was very likely afforded by the newly-formed epithelial layer. This protective mechanism, the "mucous barrier," as it has been called by Farrell,[6] Ivy,[7] and Hollander,[8] may be of greater importance in the pathogenesis of gastric ulcer than previously considered.

Conclusions. 1. Changes in the gastric mucosa produced by continuous irritation are described.

2. The sequence of events following irritation is compared in colon, small bowel, and stomach.

3. The process of regeneration after superficial destruction of the mucosa is described and is offered as a possible simple explanation for the resistance of the stomach to auto-digestion.

4. The possible implication of this mechanism in the pathogenesis of gastric ulceration is suggested.

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[3] Whitlow, J. E., Thesis, M.S., 1920, Loyola Univ. Med. School.

[4] Dragstedt, L. R., and Vaughn, A. M., *Arch. Surg.*, 1924, *8*, 791.

[5] Varco, R. L., and Wangensteen, O. H., unpublished observations, 1940.

[6] Farrell, J. T., *Am. J. Phys.*, 1928, *85*, 672.

[7] Ivy, A. C., *J. Nat. Cancer Inst.*, 1945, *5*, 313.

[8] Hollander, F., Stein, J., and Lauber, F. U., *Gastroent.*, 1946, *6*, 576.

16951

Effect of DDT on Functional Development of Larvae of *Rana pipiens* and *Fundulus heteroclitus.*

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The effect of DDT (1-trichlor-2, 2-bis-p-chlorophenyl ethane) on adult fish, frogs,[1] and mammals,[2] has been reported recently. The general results indicate that DDT specifically affects the nervous system, a fact previously demonstrated in studies on insects. It seemed desirable to determine the stage in early embryonic development when DDT first affects the central nervous system. For this study poikilothermous and aquatic forms were chosen, namely the embryos of the frog *Rana pipiens* and the fish *Fundulus heteroclitus.*

Materials and methods. A total of 400 larvae of *Rana pipiens* and 250 of *Fundulus heteroclitus* were used in this study. The larvae were introduced into the experimental medium at various stages of development, for varying periods. The stages of *Rana pipiens* are those established by Shumway[3] and for *Fundulus heteroclitus* by Oppenheimer.[4]

The experimental medium consisted of a saturated solution of DDT in spring water for Rana and in distilled water for Fundulus (the latter being able to survive well in distilled water). DDT is quite insoluble but it is estimated that when 4 parts per million are used, that at least 1 part per million goes into solution.

Rana pipiens larvae were provided with 50 cc of medium for 25 larvae in a 250 cc finger bowl while *Fundulus heteroclitus* larvae were placed 50 to a finger bowl of 75 cc of medium. The experimental media were changed daily to keep the concentration at the experimental level and to avoid chemical deterioration. The controls were treated in an identical manner except for the use of DDT. Laboratory temperatures of 23°C to 25°C were used for all larvae.

The response to stimulation was measured by the Detwiler[5] "race-track" method. The photographs were taken with a Mifilmca Adapter, through the low magnification microscope, on Microfile film and were developed in Microdol developer.

Experimental data. A. *Growth Rate. Rana pipiens*: Frog larvae placed in the experimental medium at stage 11 and left for 7 days showed no appreciable change in growth rate. By the 9th day of exposure, however, the experimentals showed an average length 2 mm less than that of the controls and on the 11th day, the experimentals had shown no further growth while the controls had continued to grow to an average of about 15 mm. Growth, therefore, was completely inhibited by 9 days after the initial exposure of the larvae to DDT.

Fundulus heteroclitus: Fish larvae were subjected to the experimental medium at stage 8 and at stage 29, were allowed to hatch and survive for 6 days beyond hatching and showed no evidence of any effect on their growth.

B. *Deformities. Rana pipiens*: Beginning the 9th day after subjection to the experimental medium of DDT, the larvae showed a narrowing of the trunk region and on the 11th day there developed a constriction just posterior to the operculum and a lateral acute bending of the tail at the point of junction with the body (Plate I, Fig. 1 to 3). These deformities were observed in the experimental larvae almost without exception. There developed a degree of respiratory paralysis in

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* Now at Radiological Research Laboratory, Columbia University.

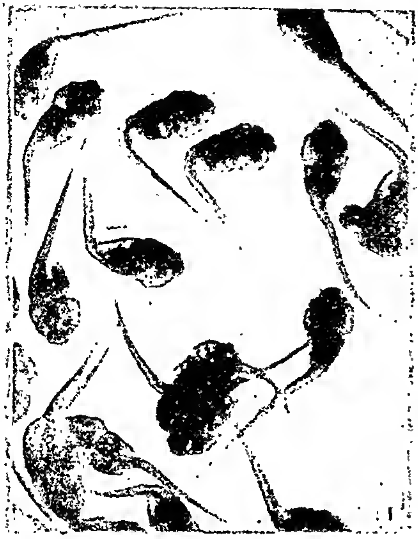
† Submitted in partial fulfillment of the requirements for the M.A. Degree at Washington Square College of New York University.

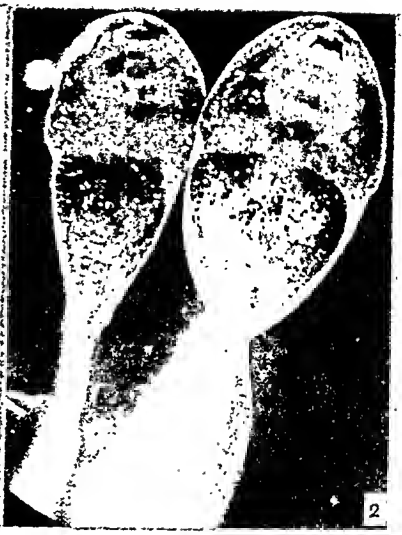
[1] Ellis, M. M. *et al.*, *Science* 1944, **100**, 477.

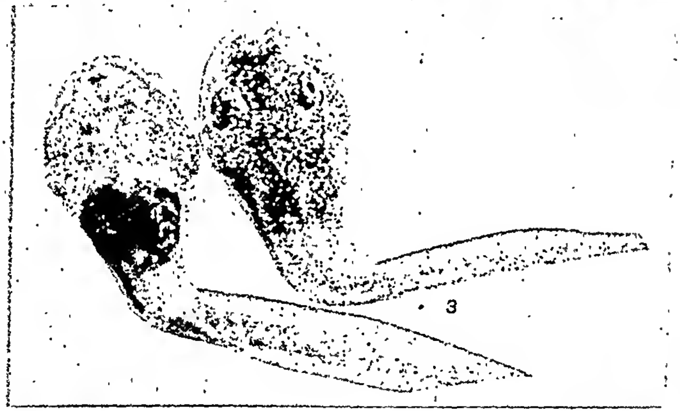
[2] Nelson, A., *et al.*, *Public Health Rep.*, 1944, **59**, 1009.

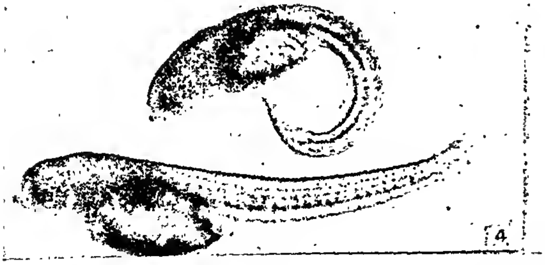
[3] Shumway, W., *Anat. Rec.*, 1940, **78**, 139.

[4] Oppenheimer, J., *Anat. Rec.*, 1937, **68**, 1.









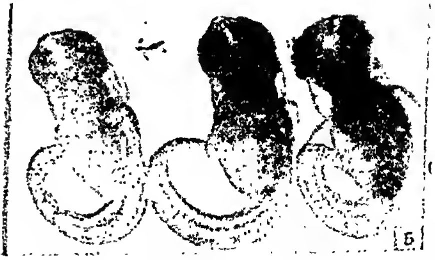


PLATE 1.

Tail curvature of *Rana pipiens* and *Fundulus heteroclitus* larvae following exposure to saturated solutions of DDT.

FIG. 1. Larvae of *Rana pipiens,* placed in a saturated solution of DDT at stages 11 and 12, after 11 days of treatment.

FIG. 2. Larvae of *Rana pipiens,* placed in a saturated DDT solution at stages 11 and 12, after 9 days of treatment. The experimental animal on the left shows an impairment of growth as compared with the control animal on the right.

FIG. 3. Higher magnification of larvae from some experimental group as larvae in Fig. 1.

FIG. 4. Larva of *Fundulus heteroclitus* (above) showing a ventral curvature of the tail upon hatching from a saturated DDT solution, in which it was immersed for 19 days, treatment beginning at stage 8; control (below) untreated.

FIG. 5. Larvae of *Fundulus heteroclitus* showing lateral curvature of tail upon hatching from a saturated DDT solution in which they were immersed for 19 days, treatment beginning at stage 8.

which the mouth was held open but the larvae showed none of the usual respiratory movements.

Larvae introduced into the experimental medium at stages 16, 18, 19 and 25 also showed these deformities but their onset was slower, the older the larvae.

Fundulus heteroclitus: Larvae introduced into the experimental medium at stage 8 hatched at the same time as the controls but upon hatching the experimentals exhibited an acute ventral bending of the tail in more than 80% of the larvae, (Plate I Fig. 4 and 5). There was also a slight constriction just below the operculum, and the mouth was held open (as with *Rana pipiens larvae*) without related respiratory movements.

When Fundulus larvae were introduced into the experimental medium at stage 29, they exhibited mild cases of lordosis and kyphosis by the 6th day post hatchings but there was no bending of the tail shown by larvae exposed earlier and for longer periods.

C. *Mortality*. The mortality rate of the experimentals was more than twice that of the controls for both *Rana pipiens* and for *Fundulus heteroclitus* larvae. Only 75 out of 400 *Rana pipiens* larvae and 35 out of 150 *Fundulus* larvae survived the treatment.

D. *Neuro-Muscular responses. Rana pipiens*: Using the race-track method of Detwiler[5] the degree of response to tactile stimulation at different stages in DDT treatment was determined. A decrease in motor activity was first noted on the 7th day of exposure, was considerably more pronounced on the 9th day and almost extinct by the 11th day (see Fig. 1). On the 11th day the responses to tactile stimulation consisted of generalized fine tremors and an occasional initial sharp single contraction of the tail musculature. There was a definite decrease in muscle tonus and in the ability to assume a normal position.

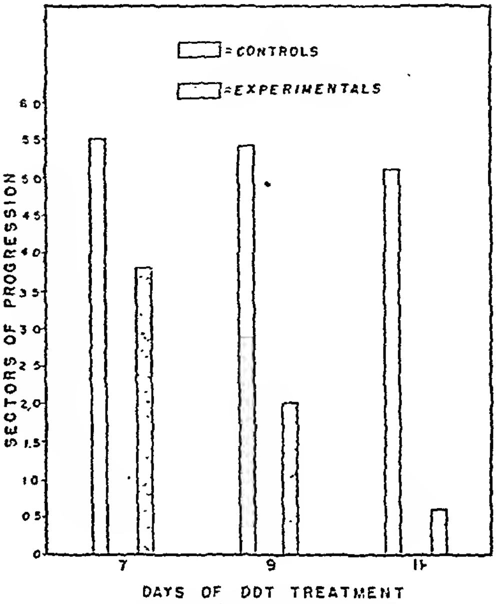


FIG. 1.

Neuro-muscular responses of *Rana pipiens* larvae to tactile stimulation following exposure to saturated solutions of DDT.

Fundulus heteroclitus: Larvae exposed to the experimental medium showed a slight decrease in motor responses to tactile stimuli as early as the fourth day after hatching, even though there was no morphological evidence of damage. There was marked decrease in the response by the 6th day, the larvae showing very much reduced activity and occasionally developing spasmodic contradictions of the trunk and tail musculature, and general fine tremors throughout the body without appreciable forward progression. There was no swimming activity.

E. *Recovery. Rana pipiens*: Larvae subjected to the DDT medium at stage 11 were removed at daily intervals from the 2nd to the 11th day after initiation of the treatment, and placed in the normal control medium. Complete recovery was effected in those larvae which were returned to the normal medium up to and including the 6th day of treatment. Beyond the 6th day of exposure, the larvae retained all of the morphological and physiological symptoms present at the time of removal from the experimental medium, but without further effects.

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[5] Detwiler S. R., *J. Exp. Zool.*, 1946, 102, 321.

Fundulus heteroclitus: Larvae which were removed from the experimental DDT medium on the 16th day after immersion at stage 8 retained the same deformities and abnormalities in behavior as the experimentals which were allowed to hatch in the DDT medium. Prior to the 16th day there was little or no retention of damage effects.

Discussion. The exact mechanism of DDT action on the living system is still unknown, except that the findings of this investigation corroborate earlier findings on higher forms that the effect is primarily on the neuro-muscular mechanism.

Ellis *et al.*,[1] and Odum and Sumerford,[6] found that cold-blooded animals are more easily affected than are the warm-blooded animals. Ellis *et al.*[1] fed DDT to goldfish in pellet form and the total mortality was approximately 55%. The fish were hyper-irritable, and then developed muscular incoordination, muscular spasms, and finally marked prostration during which time the fish lay on its side, breathing irregularly and manifesting convulsive movements. These symptoms were also found in the present investigation in the larvae of *Fundulus heteroclitus*.

The dose required to produce symptoms of toxicity varies with different animals. Odum and Sumerford,[6] found that the median lethal dose for goldfish was 0.1 ppm, for Gambusia was 0.01 ppm and for Culex larvae was 0.0001 ppm. Ginsburg,[7] stated that mosquito larvae fed on DDT were not poisonous to goldfish, but that DDT in solution was definitely toxic.[7] Concentrations of DDT at 1 part in 10,000,000 caused 40% mortality while 1/20,000,000 seemed to be non-toxic.

The concentration of DDT in solution is very difficult to regulate or determine. It was found possible to increase the concentration to 4 ppm by treating the mixture ultrasonically, but the control medium, also treated ultrasonically, was somewhat toxic to the larvae. Based upon rough calculations in relation to the amount introduced and the amount undissolved, and to observations of others, it was estimated that the concentrations used amounted to at least 1 ppm. For purposes of duplication of experimental procedures, however, it can be stated that the concentrations used were saturated at laboratory temperatures of 23° to 25°C, and that this probably represented 1 ppm.

Recently Oppenheimer[8] found similar damaging effects on the larvae of *Fundulus heteroclitus* following subjection to dilute solutions of metrazol. The sharply angled tails appeared as identical morphological effects of DDT and metrazol, and it is entirely possible that the two compounds effect the neuromuscular apparatus of the larvae in much the same manner.

Conclusions. 1. A saturated solution of DDT (estimated at 1 ppm) in spring water for *Rana pipiens* larvae and in distilled water for *Fundulus heteroclitus* larvae, was sufficiently toxic to produce morphological deformities of a similar nature, the most obvious of which was the sharply bent tail and the very much reduced neuro-muscular response to tactile stimulation.

2. The initial evidence of damage by DDT appeared after 7 days of treatment of *Rana pipiens* larvae (beginning at stage 11) and after hatching of *Fundulus heteroclitus* larvae (treatment beginning at stage 8). There was a considerable lag in the effect, due, in all probability, to the fact that DDT is relatively insoluble and therefore difficult to assimilate by the larvae.

3. In addition to the bent tail, there was a gradual decrease in neuro-muscular responses as determined by the Detwiler racetrack method following tactile stimulation, in both Rana and Fundulus larvae. Ultimately the larvae responded with only muscular tremors.

4. The toxic symptoms were not manifest in Rana larvae if they were returned to the control medium after 6 days of subjection to

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[6] Odum, E. P., and Sumerford, W. T., *Science*, 1946, **104**, 480.

[7] Ginsburg, J. M., *J. Econ. Ento.*, 1945, **40**, 475.

[8] Oppenheimer, J. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **66**, 49.

the DDT or in Fundulus as late as 16 days in the medium if subjected at stage 8.

5. There was no effect of DDT on the growth rate of Fundulus larvae, but after 9 days of exposure of Rana larvae they show a definite inhibition of growth.

6. The older the larvae at the time of DDT exposure, the longer the period before the onset of deleterious symptoms.

16952

Biochemical Studies on Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.*

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In a recent communication[1] it was shown that the apparent xanthine oxidase activities of livers from chicks fed a purified diet were inversely related to the pteroylglutamic acid (PGA) content of the diet. The livers from the same experimental chicks were also studied for their nucleic acid and PGA content and for their apparent conjugase activity; the values so obtained are reported here.

Experimental. The diets and the methods of handling and care of the chicks are given fully in the previous report.[1] PGA was determined microbiologically by use of *Streptococcus faecalis*.[2] Conjugase was determined by the method of Laskowski, Mims, and Day[3] using Difco yeast extract as the substrate. Since there may be several conjugase inhibitors[4-7] in a mixture such as that used in these tests the absolute values may not be valid, but there seems to be no reason why such a method is not satisfactory for comparative purposes when using a single batch of substrate and the same organ from each bird.

Pentose nucleic acid and desoxypentose nucleic acid were determined on aliquots of 1:5 liver brei employing the methods outlined by Schneider.[8,9]

Results and discussion. The results of the nucleic acid determinations are given in Table I. As indicated earlier,[1] the livers of chicks were relatively larger when the diet was PGA-deficient. The data are arranged to show the nucleic acid content per gram of liver, the total nucleic acid per chick, and the amount per 100 g of chick. The values ob-

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[1] Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, 1948, **176**, 1095.

[2] Mitchell, H. K., and Snell, E. E., Univ. of Texas Pub., 1941, No. 4137, 36.

[3] Laskowski, M., Mims, V., and Day, P. L., *J. Biol. Chem.*, 1945, **157**, 731.

[4] Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J., *J. Biol. Chem.*, 1946, **163**, 649.

[5] Sims, E. S., and Totter, J. R., *Fed. Proc.*, 1947, **6**, 291.

[6] Mims, V., Swenseid, M. E., and Bird, O. D., *J. Biol. Chem.*, 1947, **170**, 367.

[7] Hodson, A. Z., *Arch. Biochem.*, 1948, **16**, 309.

[8] Schneider, W. C., *J. Biol. Chem.*, 1945, **161**, 293.

[9] Schneider, W. C., *Cold Spring Harbor Symposia on Quantitative Biology*, 1947, **12**, 169.

TABLE I.

Desoxypentose Nucleic Acid and Pentose Nucleic Acid in Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.

Diet	No. of chicks	Liver desoxypentose nucleic acid				Liver pentose nucleic acid			
		Avg, mg/g	Range, mg/g	Per chick, mg	Per 100 g chick, mg	Avg, mg/g	Range, mg/g	Per chick, mg	Per 100 g chick, mg
Basal	14	2.5	2.1-3.5	14.6	9.6	6.0	3.2-7.8	38.6	27.3
'' + 5 μg PGA/100 g	4	3.2	2.9-3.9	20.0	11.3	6.8	5.4-8.4	41.6	34.3
'' + 10 '' '' ''	8	3.0	2.3-3.7	22.1	8.1	7.6	4.9-9.9	56.8	35.8
'' + 20 '' '' ''	7	2.9	2.2-3.8	23.1	5.5	6.9	5.0-8.2	55.4	26.6
'' + 40 '' '' ''	9	3.1	2.0-4.9	26.7	4.3	6.5	4.3-7.9	58.8	27.4
'' + 80 '' '' ''	9	3.3	2.4-4.2	30.2	4.8	7.0	4.8-8.0	65.3	23.4
'' + 200 '' '' ''	8	3.1	2.1-4.6	34.3	3.8	6.5	4.6-7.9	70.5	23.1
'' + 1000 '' '' ''	8	3.3	2.9-3.7	27.5	4.6	6.9	3.9-8.5	58.6	17.8
Commercial diet*	8	3.5	2.6-3.8	28.0	4.4	6.8	4.9-8.4	56.0	17.3

* Purina "Startena," containing 175 μg of total PGA per 100 g, as shown by assay with *S. faecalis* and suitable conjugases.

tained agree satisfactorily with those obtained by Schneider on rat liver.[6]

The possibility that pteroylglutamic acid may be necessary for thymine synthesis and presumably, therefore, for synthesis of thymine-containing nucleic acids is emphasized in current theories concerning mechanism of action of this vitamin.[10-12] It is of special interest therefore to compare the thymonucleic (desoxypentose nucleic) acid contents of the livers of chicks on the basal diet with those receiving adequate amounts of PGA. As seen from Table I, there is a somewhat lowered desoxypentose nucleic acid content per gram of deficient chick liver as compared with livers from the other groups. However, groups receiving amounts of PGA entirely inadequate (5 μg, 10 μg) for prevention of deficiency symptoms nevertheless have values of desoxypentose acid per gram of liver equal to or greater than those receiving more than enough of the vitamin. When calculated on the basis of total liver nucleic acid per 100 g of chick, those on the basal diet have greater amounts than the ones receiving excess of PGA (200 μg, 1000 μg). If it may be assumed that the method used is specific for thymine-containing nucleic acids, the data do not offer strong support to the view that PGA deficiency blocks the synthesis of thymine. In this connection it may be recalled that Davidson[13] has found that both types of nucleic acid are elevated in the bone marrow of untreated pernicious anemia patients. Successful treatment of his patients was followed by a reduction to normal of their marrow nucleic acids.[13] It is difficult to reconcile these results with the hypothesis that the sole action of PGA is to promote thymine synthesis.

The pentose nucleic acid content was more uniform than the desoxypentose nucleic acid content. The level of pentose nucleic acid in the livers of the totally deficient groups was

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[10] Stokes, J. L., *J. Bact.*, 1944, **47**, 433.

[11] Spies, T. D., Vilter, C. F., Cline, J. K., and Frommeyer, W. B., *Southern Med. J.*, 1946, **39**, 269.

[12] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

[13] Davidson, J. N., *Cold Spring Harbor Symposia on Quantitative Biology*, 1947, **12**, 50.

TABLE II.

Average Contents of Pteroylglutamic Acid and Conjugase in the Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.

		Free PGA autolyzed at		Total PGA		Apparent liver conjugase activity*	
	No. of chicks	pH 4.5 µg/g	pH 7.0 µg/g	Hog kidney conjugase pH 4.5 µg/g	Chicken pancreas conjugase pH 7.0 µg/g	pH 4.5 units/g	pH 7.0 units/g
Basal	12	2.0	1.5	2.1	1.9	3.1	6.4
,, + 5 µg PGA/100 g	4	1.3	1.5	1.1	1.5	2.9	7.4
,, + 10 ,, ,, ,,	8	1.3	2.0	2.5	3.1	4.2	12.3
,, + 20 ,, ,, ,,	7	2.2	2.2	3.2	3.0	3.7	10.6
,, + 40 ,, ,, ,,	9	2.9	2.3	2.9	3.2	2.7	6.3
,, + 80 ,, ,, ,,	9	8.5	8.7	9.9	8.6	4.1	8.9
,, + 200 ,, ,, ,,	9	5.0	5.1	6.0	6.3	5.1	12.5
,, + 1000 ,, ,, ,,	8	7.7	7.3	7.8	8.9	3.3	8.2
Commercial diet†	8	6.7	5.3	7.3	7.1	2.1	6.2

* A unit of conjugase activity is expressed here as the µg of PGA liberated in 4 hours by 0.2 ml of 1:20 liver brei acting on 80 mg of Difco yeast extract in a total volume of 2 ml.

† Purina "Startena," containing 175 µg of total PGA per 100 g, as shown by assay with *S. faecalis* and suitable conjugases.

only slightly lowered when compared with the other groups; the difference is probably not statistically significant.

According to Mims *et al.*,[6] desoxypentose nucleic acid is a potent inhibitor of conjugase activity. It might therefore be supposed that any alterations in the level of this nucleic acid would be reflected inversely by changes in the conjugase activity of the liver samples. In Table II are shown the apparent conjugase values at pH 7 and pH 4.5 as well as the free and total PGA content of the livers; the total PGA was determined with chicken pancreas conjugase at pH 7 and hog kidney conjugase at pH 4.5. There seems to be no particular relation between the conjugase activity and either the PGA intake or the desoxypentose nucleic acid content of the livers. It is interesting that the only group of chicks receiving conjugated PGA, those on the commercial diet, exhibited the lowest liver conjugase activity. The degree of reduction, however, is of doubtful significance.

The free PGA content of the livers appears to be about equal to the total PGA, *i.e.* little additional vitamin was liberated by treatment with added conjugase from either chicken pancreas or hog kidney. In a few cases the free is shown as greater than total PGA; these occurrences are undoubtedly due to cumulative errors in the assays.

The average contents of PGA in the livers of the various groups provide evidence from which the minimum requirement for the vitamin may be estimated. It is interesting that the liver contents of PGA in the chicks up to the group receiving 40 µg per 100 g of diet show little change, while the 80 µg group and those receiving more than 80 µg are 2 to 3 times higher than the deficient groups in this respect. From this it appears that the minimum requirement for protection from deficiency should be set at a value not lower than 40 µg per 100 g of diet. This is in agreement with the estimate from the growth curve of these chicks published earlier.[1] Estimation from blood cell numbers, however, indicates the minimum value to be somewhat lower when calculated for red cell values. On the contrary, white cell (chiefly lymphocytes)[14,15] numbers and thymus weights[15] increased logarithmically with increasing levels of PGA to as high as 200 μg per 100 g of diet.

The concentrations of PGA in the livers of the chicks also provide some evidence concerning the mode of action of the vitamin in reducing the apparent liver xanthine oxidase activity. *In vitro* tests of the sample of PGA used in these experiments indicated that a concentration of approximately 40 μg per ml would be needed to reduce the xanthine oxidase activity to one-half its initial value.[†] The relatively low levels of PGA encountered in all of the liver samples indicate that either the impurity probably responsible for the *in vitro* anti-xanthine oxidase activity[16] is better retained than is PGA, or that some other mechanism than the accumulation of this substance is responsible for the reduced activity found in our previously published experiments.[1] It is conceivable that the xanthine oxidase is inhibited or reduced by a metabolic product of PGA normally produced in relatively large quantity.

Summary. Determinations of desoxypentose nucleic acid and pentose nucleic acid, pteroylglutamic acid (PGA), and conjugase have been made on the livers of chicks receiving 0, 5, 10, 20, 40, 80, 200, and 1000 μg of PGA per 100 g of diet and on controls receiving a commercial diet. Desoxypentose nucleic acid was found to be somewhat low in the livers of the negative control group of chicks but differences between all other groups were of doubtful significance.

The liver conjugase levels of the various groups, whether determined at pH 4.5 or at pH 7.0, were found to be unrelated to the dietary intake of PGA and to the nucleic acid content of the livers.

The liver PGA freed by autolysis was approximately the same as that determinable after treatment with either chick pancreas conjugase or hog kidney conjugase. The levels of PGA found in the chick livers indicate that storage of excess vitamin did not take place until the dietary level exceeded 40 μg per 100 g.

The bearing that the findings have on the mode of action of dietary PGA in reducing liver xanthine oxidase is briefly discussed.

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[14] Campbell, C. J., McCabe, M. M., Brown, R. A., and Emmett, A. D., *Am. J. Physiol.*, 1945, **144**, 348.

[15] Unpublished data from this laboratory.

[†] Experiments conducted *in vitro* indicate that 2 mols of the xanthine oxidase inhibitor react with one mol of chick liver xanthine oxidase while only one mol reacts per mol of rat liver xanthine oxidase. That is, the degree of inhibition using chick liver is proportional to the square of the concentration of added PGA while it is proportional to the logarithm of the PGA concentration when using rat liver as the source of xanthine oxidase.

[16] Kalckar, H. M., Kjelgaard, N. O., Klenow, H., *J. Biol. Chem.*, 1948, **174**, 771.

16953 P

Influence of Pteroylglutamic Acid Administration Upon Fecal Riboflavin Values in Human Subjects.*

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In the course of investigations in this laboratory on the metabolism of riboflavin in human subjects on weighed, uniform diets of

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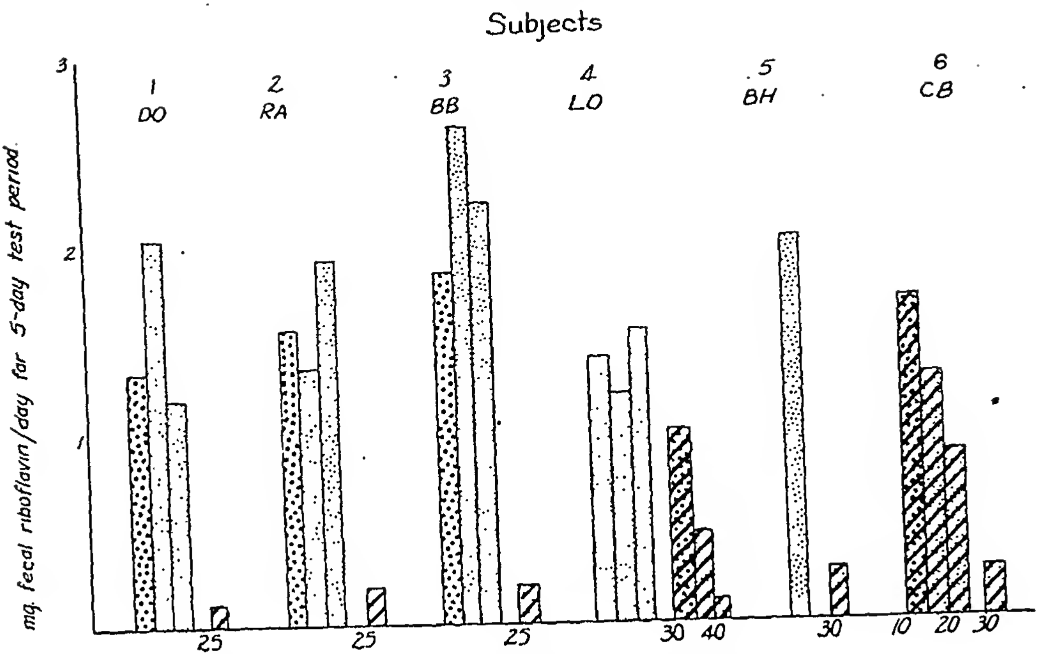

FIG. 1.

The progressively depressing effect of the daily administration to human subjects of 10 mg doses of pteroylglutamic acid on fecal riboflavin values.

Small dots: basal diet; larger dots: self-chosen diet; ////: 10 mg dose pteroylglutamic acid; numerals on the abscissa indicate number of days dose prior to fecal collection.

known riboflavin content[1-3] doses of pteroylglutamic acid were incorporated in certain of the experimental diets.

Experimental. Six university women in a satisfactory nutritional state were maintained on an adequate uniform weighed basal diet providing a daily intake of 3.8 mg of riboflavin with and without the addition of 10 mg of synthetic pteroylglutamic acid. This dose is considered to be within the lower part of the therapeutic range.[4] For the first two basal periods of Subject 4, the diet was identical with one reported earlier[1] and for all other subject-periods this diet was modified only by the addition of 50 g of meat loaf, thus increasing the protein content of the diet to 84 g. For securing additional data, fecal riboflavin values for 5-day periods were also determined on self-chosen diets.

For conformity with other studies, certain supplements were added to the basal diet[‡] as follows: Subjects 1, 2 and 4 received 14 g dried brewers' yeast daily, and Subjects 3, 4 and 6 received supplements[‡] of 2.0 mg thiamine, 0.45 mg riboflavin, 5.24 mg niacin, 50.4 mg choline, 0.14 mg pyridoxine, 1.0 mg calcium pantothenate, and 10 μg pteroylglutamic acid with the exception of the first 2 periods of Subject 4.

Five-day fecal collections were homogenized in a Waring Blendor and preserved in acid-alcohol. Riboflavin was determined by the fluorometric method of Conner and Straub.[5] Duplicate determinations were made

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[†] Present address: Department of Home Economics, University of California, Berkeley, Calif.

[1] Price, E. L., Marquette, M. M., and Parsons, H. T., *J. Nutrition*, 1947, **34**, 311.

[2] Price, E. L., M. S. Thesis, 1946, Univ. Wis.

[3] Otto, D. M., M. S. Thesis, 1948, Univ. Wis.

[4] Spies, T. D., *Annual Rev. Biochem.*, 1948, **17**, 449.

[‡] Thanks are due to the Lederle Laboratories, Inc., Pearl River, N. Y., for the synthetic pteroylglutamic acid (Folvite); to Hoffman La Roche, Nutley, N. J., for other vitamin supplies; and to the Pineapple Research Institute of Hawaii for the gift of specially packed crushed pineapple.

[5] Conner, R. T., and Straub, G. J., *Ind. Eng. Chem. Anal. Ed.*, 1941, **13**, 385.

on separate days for all samples. Food aliquots representing 25% or 50% of the weighed daily food intake, except the self-selected diets, were homogenized and assayed for riboflavin.

Results and discussion. The fecal riboflavin values observed during the control periods are consistent with other data from this laboratory for subjects ingesting essentially the same basal diet. Similar fecal riboflavin concentrations are also reported by Hathaway and Lobb[6] for subjects ingesting a natural diet containing 1.33 mg of riboflavin.

Upon supplementation of the diets of the subjects with 10 mg of pteroylglutamic acid daily for periods of 25 to 40 days, regular and consistent decreases were observed in fecal riboflavin. Values comparable to the lower range in this series have been noted in this laboratory only in one other instance, *i.e.*, when subjects were consuming a diet composed of milk with ascorbic acid and mineral supplements.[7]

Darby *et al.*[8] have noted that certain deficiency states due to decreases in gastrointestinal absorption may be relieved by the administration of pteroylglutamic acid. These observations suggest that in the present experiment, certain vitamin interrelationships involving the absorption of riboflavin might be concerned in the decrease in fecal riboflavin in the presence of long-continued daily dosage of 10 mg of pteroylglutamic acid. Another obvious possibility is that administration of pteroylglutamic acid measurably influences the character of the intestinal flora, resulting in a decrease in riboflavin synthesis, an increase in riboflavin destruction or both. The nature of this pronounced change in fecal riboflavin is being investigated; these results are being reported at this time because of their possible clinical implications.

Summary. As a part of long-time experiments in this laboratory on riboflavin metabolism in human subjects on weighed diets of known riboflavin content, it was noted that with the daily administration of 10 mg of synthetic pteroylglutamic acid for 25 to 40 days there was a striking decrease in fecal riboflavin values as compared to basal periods.

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[6] Hathaway, M. L., and Lobb, D. E., *J. Nutrition*, 1946, **32**, 9.

[7] Gardner, J., Neal, A. L., Peterson, W. H., and Parsons, H. T., *J. Am. Dietet. Assn.*, 1943, **19**, 683.

[8] Darby, W. J., Jones, E., Warden, H. F., and Kaser, M. M., *J. Nutrition*, 1947, **34**, 645.

16954

Effect of Adrenal Cortical Extract on the Blood Picture and Serum Proteins of Fowl.

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Preliminary to studies on the effects of adrenal cortical extract (ACE) on embryonic development of the chick's blood, it became desirable to know how the blood elements of the adult fowl respond to cortical substances. We have been unable to find pertinent data for the fowl; furthermore the results obtained with various mammals are not consistent. Absolute peripheral lymphopenia has been observed in the normal mouse,[1] rat,[1,2,3] rabbit,[1] dog,[2] and in man[4,5] after injections of pituitary adrenotrophic hormone, and in the mouse,[1] rat,[1,6] rabbit,[1] and man[1,4,5] after adrenal cortical substances. However, others[3,7] have reported little or no change in the blood cell picture of the rabbit after therapy with ACE. The normal rat[8] and cat[9] have been shown to respond to pituitary adrenotrophic hormone therapy with a marked decline in number of thoracic duct lymphocytes. On the other hand Valentine *et al.*[10] observed no change in number of thoracic duct lymphocytes in cats injected with ACE.

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[1] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

[2] Reinhardt, W. O., Aron, H., and Li, C. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **57**, 19.

[3] Yoffey, J. M., and Baxter, J. S., *J. Anat.*, 1946, **80**, 132.

[4] Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrinology*, 1948, **8**, 15.

The relationships of hypercortico-adrenal activity to serum protein levels in mammals is likewise controversial. Dougherty and White[11] report such activity to be associated with elevation in total serum protein in mice and rats. In the rabbit there is an increase in serum globulin due to rise in beta and gamma globulin.[12] In rats a decrease in gamma, but an increase in alpha and beta globulin, after ACE therapy has been reported.[13] Li and Reinhardt[14] found no effect of pituitary adrenotrophic hormone on plasma globulins in the normal rat, and Eisen *et al.*[15] detected no change in gamma globulin concentration after repeated injections of ACE, although a single injection did raise the serum antibody nitrogen in immunized rabbits.

It is our purpose in the present paper to report changes in the blood picture of 2 breeds of fowl at various periods after a single injection of ACE. Some observations on serum protein levels are included.

Method. Adult Single Comb White Leghorn laying hens and New Hampshire Red non-laying pullets were used. The Leghorns were 7 months of age at the outset of the experiment and weighed 1800 to 2200 g. The pullets were 3½ months old and weighed 1800 to 2200 g. The animals were housed outdoors in wire bottom cages protected from wind, dampness, and rain, and were maintained on commercial diets.

Blood was obtained from the ulnar vein and the smears were usually stained within 24 hours using Wright's and Giemsa, the latter diluted tenfold. The differential white blood cell determinations were made by counting 300 leucocytes, and occasionally 400, in representative regions of each slide.

Blood for both total leucocyte and erythrocyte counts was diluted 100 times[16] in a certified red blood cell diluting pipette with a solution[17] composed of 25 mg of neutral red added to 100 ml of 0.9% saline, and the cells were identified in a Neubauer Brightline Counting Chamber.

Total protein of the sera was estimated with the biuret reagent of Weichselbaum.[18] Optical densities of the sera were determined at 555 mu on a model 11A Coleman spectrophotometer by subtracting the optical density readings of serum-containing cuvettes from those with saline prepared at the same time. The density readings are thus simply measures of the relative total protein content rather than of absolute amounts.

Precipitation of total serum globulin was carried out with the ammonium sulfate method of Fine.[19] Nine and a half ml of 27.79% ammonium sulfate was added to 0.5 ml of serum placed in a 15 ml conical centrifuge tube, giving a final concentration of 2M ammonium sulfate. The tubes were refrigerated overnight and centrifuged the next

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[5] Hills, A. G., Forsham, P. H., and Finch, C. A., *Blood*, 1948, **3**, 755.

[6] Nichols, J., and Miller, A. T., *Science*, 1948, **108**, 378.

[7] Fox, C. A., and Whitehead, R. W., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 756.

[8] Reinhardt, W. O., and Li, C. H., *Science*, 1945, **101**, 360.

[9] Yoffey, J. M., Reiss, M., and Baxter, J. S., *Nature*, 1946, **157**, 368.

[10] Valentine, W. N., Craddock, C. G., and Lawrence, J. S., *Blood*, 1948, **3**, 729.

[11] Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 26.

[12] White, A., and Dougherty, T. F., *Endocrinology*, 1945, **36**, 207.

[13] Gjessing, E. C., and Chanutin, A., *J. Biol. Chem.*, 1947, **169**, 657.

[14] Li, C. H., and Reinhardt, W. O., *J. Biol. Chem.*, 1947, **167**, 487.

[15] Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R-R., and Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 301.

[16] Wetmore, W. W., *Science*, 1940, **92**, 386.

[17] Forkner, C. E., *J. Exp. Med.*, 1929, **50**, 121.

[18] Weichselbaum, T. E., *Am. J. Clin. Path., Tech. Bull.*, 1946, **10**, 40.

[19] Fine, J., *Biochem. J.*, 1935, **29**, 799.

TABLE I. Effect of a Single Injection of 2 ml of ACE* on Blood Cell Levels in 11 White Leghorn Laying Hens.

	Hr	Cells per cu. mm				%				
		RBC × 1000	WBC	Lymphocyte	Polymorph	Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil
Mean	Pre-0†	2,714	27,118	20,908	4,068	77.1	7.9	1.5	1.6	11.9
S.D.‡		243	4,288	3,109	1,792	4.8	2.1	0.6	0.8	5.0
Mean	0	2,738	30,727	23,352	5,715	76.0	5.4	0.8	1.1	16.7
S.D.		325	5,656	5,098	2,794	8.4	2.0	0.7	0.9	7.0
Mean	1	2,448	23,383	17,257	4,887	73.8	5.3	0.3	1.4	19.2
S.D.		274	6,862	5,664	3,132	7.9	2.4	0.3	0.9	7.6
Mean	3	2,367	18,378	11,817	5,440	64.3	6.1	0.4	1.5	27.7
S.D.		256	7,061	5,131	3,173	11.4	2.4	0.5	1.1	11.3
Mean	6	2,492	26,879	17,794	7,768	66.2	4.9	0.4	1.2	27.3
S.D.		238	6,967	5,843	2,858	10.0	2.5	0.4	0.9	9.1
Mean	9	2,588	30,107	21,737	7,165	72.2	4.0	0.5	0.8	22.5
S.D.		300	6,163	5,471	3,235	11.3	1.5	0.5	0.6	9.4
Mean	24	2,624	29,573	23,274	4,968	78.7	4.5	0.7	1.6	14.5
S.D.		447	5,875	4,562	3,247	10.4	2.2	0.6	1.2	8.4

* Wilson's product injected into 9 hens and Upjohn's into 2 hens.

† Derived from 40 counts made on the same 11 hens during the 4 weeks preceding injection.

‡ Standard deviation of the mean; S.D. = $\sqrt{\frac{\Sigma d^2}{n-1}}$

morning at 2500 r.p.m. for ½ hour. The supernatant, containing mainly albumin and ammonium sulfate, was separated from the packed precipitate by decantation, and the tubes were inverted for 1½ hours on filter paper to remove excess liquid. The globulin was dissolved in 0.3 ml of 0.85% saline. To 1/10 ml of this protein solution in a cuvette were added 4.9 ml of 0.85% saline and 5.0 ml of biuret reagent. Optical density readings were made at 555 mu on the spectrophotometer. Each serum sample was precipitated and tested in duplicate.

The adrenal cortical extracts (aqueous)* and sterile 0.85% saline were injected subcutaneously in the pectoral region. Immediately prior to the injections blood counts were taken from each animal and are designated in the tables as "0 hour" counts.

Experiments and Results. (1) *White Leghorn laying hens.* A single injection of 2 ml of ACE (Table I) was followed by a marked leucopenia, pronounced absolute lymphopenia, decrease in number of erythrocytes and an absolute polymorphonuclear leucocytosis. The severe lymphopenia, indicated by a post-injection mean of ½ the pre-injection mean at 0 hour accounts for the leucopenia. Differential counts revealed a 11.7% decrease in lymphocytes and a concomitant increase of 11% in polymorphonuclear cells 3 hours following injection. The latter change was apparently due mainly to heterophils. All changes were maximal at 3 hours after injection except for the absolute granulocyte alteration, which was maximal at 6 hours. The blood picture returned to normal by the end of 24 hours. The absolute and differential alterations are statistically significant; furthermore all animals showed the absolute changes, and only one hen failed to reveal the percentage deviations.

The effects of saline injections, of a 24-hour fast (with water *ad libitum*), and of fasting plus saline are shown in Table II. These procedures caused no appreciable alteration in the blood picture. The effect of fasting

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* We are indebted to Dr. David Klein of the Wilson Laboratories and to Dr. W. F. Wenner of the Upjohn Company for supplying us with their adrenal cortical products.

TABLE II.

Effects of *Saline*, *of Fasting*, of Saline After Fasting, and of ACE* After Fasting on the Blood Picture of White Leghorn Laying Hens.

	No. of *hens*	Hr.	Cells per mm3				%				
			RBC × 1000	WBC	Lymphocyte	Polymorph	Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil
Inj. of 2 ml of saline											
Mean	3	0	2,507	26,917	20,457	5,087	76.0	5.1	2.1	1.3	15.5
S.D.			249	4,653	3,253	1,274	3.0	1.0	0.9	0.8	3.9
Mean		3	2,703	24,917	19,012	4,460	76.3	5.8	1.7	1.9	14.3
S.D.			255	4,404	2,244	1,630	4.2	1.1	1.2	0.9	2.3
Mean		6	2,497	26,667	20,240	4,987	75.9	5.4	1.3	1.1	16.3
S.D.			255	5,125	3,778	1,708	4.7	0.8	0.6	0.6	4.4
Mean		9	2,635	25,167	18,322	5,863	72.8	3.9	1.7	1.2	20.4
S.D.			320	1,258	702	1,784	6.3	0.8	2.5	0.6	3.3
After 24-hr fast											
Mean	10	0	2,651	26,000	18,850	5,330	72.5	7.0	1.1	1.6	17.8
S.D.			320	5,678	4,286	2,833	10.8	2.9	0.5	0.8	9.0
Mean		24	2,730	25,500	17,570	5,016	68.9	7.9	1.2	2.0	20.0
S.D.			369	5,793	4,521	3,246	12.7	4.6	0.6	1.6	9.6
Inj. of 3 ml of saline after fasting											
Mean	3	0	2,670	23,583	16,720	5,613	70.9	5.3	1.6	2.0	20.2
S.D.			255	1,808	2,234	573	4.3	1.9	1.4	1.2	4.4
Mean		3	2,730	22,750	15,971	5,642	70.2	5.0	1.1	2.1	21.6
S.D.			152	2,750	3,783	1,785	9.9	1.8	1.1	0.2	6.3
Inj. of 3 ml of ACE after fasting											
Mean	10	0	2,334	23,000	17,204	4,623	74.8	5.1	0.9	1.7	17.5
S.D.			326	4,452	4,393	1,396	7.4	1.8	0.7	1.3	6.0
Mean		3	2,270	10,450	5,706	4,096	54.6	6.2	0.2	1.1	37.9
S.D.			263	2,020	1,704	1,075	11.3	2.7	0.3	0.8	10.3

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* Product of Wilson Laboratories.

was studied since it was considered desirable to reduce the high lipid content of the sera[20] for electrophoretic analyses.

Two months after the above injection of 2 ml of ACE, the same group of birds was fasted for 24 hours and then given 3 ml of ACE (Wilson). Cell count alterations were similar to those obtained 3 hours after the first injection of 2 ml of ACE except that there was no significant decrease in number of erythrocytes (Table II). The lymphocyte mean was ⅓ the pre-injection mean. In one hen showing an absolute lymphopenia involving a drop from 22,400 to 5,490, an electrophoretic analysis (using veronal buffer)† of the serum showed no significant change in any protein fraction from levels found in the same animal's serum after saline injection 3 days earlier. However, the post-extract serum showed a 15% total serum protein increase.†

(2) *New Hampshire non-laying pullets.* A single injection of 3 ml of saline into 12 New Hampshire pullets did not alter the peripheral blood cell picture significantly (Table III). Ten days after the saline injection, each animal received a single injection of 3 ml of ACE. Three hours after injection there was an absolute lymphopenia (Table III) with a mean of 12,833 compared to the pre-injection mean of 20,466 and a slight drop in the number of erythrocytes. The total leucocyte level remained normal as a result of increase in the number of granulocytes. Differential leucocyte determinations indicate a mean decline of 27.9% in lymphocytes, an increase of 26.3% in polymorphonuclear cells, mainly due to heterophils, and a slight drop in eosinophils.

Although the aforementioned changes in blood cell values are statistically significant, data for total protein variation between post-saline and post-extract levels (Table III) yields a t value of 2.0 which is slightly below the criterion of significance (2.2). If the protein values for individual birds are considered

TABLE III. Effect of 3 ml of ACE (Wilson) on Blood Counts, Total Serum Protein, and Total Globulin Levels in 12 New Hampshire Red Non-laying Pullets (treated 10 days earlier with 3 ml of saline).

			Cells per mm3				%					Serum optical density reading	
		Hr.	RBC × 1000	WBC	Lymphocyte	Polymorph	Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil	Total protein	Total glob.
Pre-sal.	Mean	0	2,432	30,208	20,511	7,884	67.9	6.0	1.8	3.0	21.3		
	S.D		344	8,818	6,188	4,005	9.8	1.7	1.4	1.1	9.5		
Post-sal.	Mean	3	2,398	28,854	18,669	8,397	64.7	6.2	1.4	3.2	24.5	.425	.200
	S.D.		196	5,303	4,917	3,667	10.6	2.2	0.6	1.2	9.3	.091	.064
Pre-ACE	Mean	0	2,679	27,000	20,466	5,184	75.8	5.0	1.2	2.3	15.7		
	S.D.		390	4,979	4,749	1,357	5.7	2.1	1.0	1.2	4.5		
Post-ACE	Mean	3	2,535	25,792	12,833	12,190	47.9	6.6	0.3	1.9	43.3	.456	.187
	S.D.		313	5,405	3,395	3,631	8.9	2.2	0.4	1.4	8.5	.087	.048

Note: t value for protein change is 2.0, and for globulin 1.2; t = 2.2 (P = .05) is significant; $t = \frac{x}{s}\sqrt{n-1}$, x = difference in means and $s = \sqrt{\frac{\Sigma(x_1 - x)^2}{n}}$ where x_1 = difference between the control and experimental value in the same animal.

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[20] Herrmann, G. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 229.

† Performed through the courtesy of Dr. John Mehl, Professor of Biochemistry at the University of Southern California School of Medicine. Total protein determined by a biuret method.

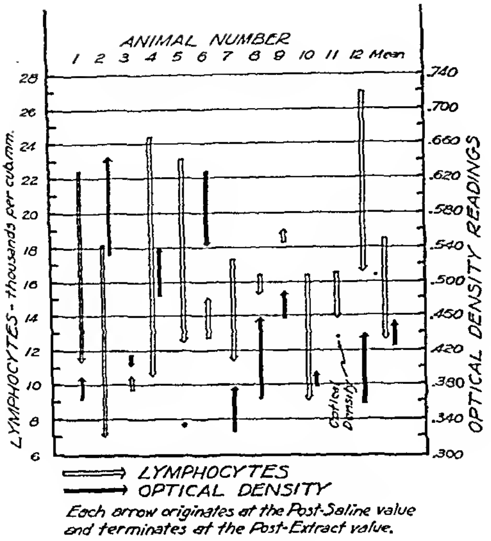

FIG. 1.
Effect of a single injection of 3 ml of ACE on lymphocyte and total serum protein levels in each of 12 New Hampshire Red non-laying pullets.

(Fig. 1), it is evident that 9 of the 12 pullets showed higher serum protein levels and one showed no change. Despite the borderline t value, this indicates a trend toward elevation in total serum protein.

It is evident from Fig. 1 that increase in serum protein levels is associated with lymphopenia. Of 9 animals revealing a lymphopenia, 8 also showed an elevation in serum protein, and one no change. Two pullets (No. 3 and 6, Fig. 1) deviated from this general picture, showing an apparent decrease in serum protein accompanied by an increase in lymphocytes. In one of these (No. 3) the changes observed are insignificant. While the results suggest a correlation between lymphopenia and serum protein increase in individual pullets, there appears to be no obvious quantitative relationship.

The t value for the difference between globulin readings was 1.2, 6 animals showing a decrease, 5 an increase, and 1 no change after extract injection. We have, therefore, no indication of a consistent globulin change as a result of ACE injection.

Discussion. The different degrees of lymphopenia obtained in the present experiments may be due to an inherent breed difference in reactivity, to differences in age since lymphoid tissue is less active in older birds,[21] or to differences in amounts of injected extract. The overall picture, however, indicates absolute and relative lymphopenia and absolute and relative heterophil leucocytosis occur consistently after a single injection of ACE into both breeds of fowl. Furthermore the maximal lymphopenic effect occurred at 3 hours in the present study on the fowl, which is in good agreement with the work on the rat and rabbit.[1]

The present results suggest an augmentative effect of ACE therapy on total protein levels in fowl. However, we can not be certain that the effect was due solely to the ACE injected inasmuch as 10 days elapsed between bleeding of animals for control and experimental serum protein determinations. The apparent increase in protein level may possibly have been due to 10 days of maturation, although no significant changes were found in the lymphocyte counts. The data of Herrmann[20] suggests that non-laying chickens of 11-22 weeks of age have a serum protein mean of 4.02 ± .5 (S.D.) as compared to 6.1 ± 1.3 for laying hens. Definite conclusions on the effects of ACE on serum protein levels must await more complete data on protein changes in the serum of the normal fowl during the 15th and 16th week of development.

Conclusions. 1. White Leghorn laying hens injected subcutaneously with aqueous adrenal cortical extract, showed leucopenia, absolute and relative lymphopenia and absolute and relative increase in granulocytes attributable mainly to heterophils. The effects were maximal at 3 hours and the blood picture returned to normal by the end of 24 hours.

2. New Hampshire Red non-laying pullets injected with ACE displayed similar changes with the exception of leucopenia which was masked by increase in polymorphonuclear cells.

3. ACE therapy produced no statistically significant increase in total serum protein or serum globulin levels. However, a trend toward increase in total serum protein, accompanied by lymphopenia, is indicated for the majority of the individual birds studied.

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[21] Biester, H. E., and Devries, L., Diseases of Poultry, The Collegiate Press, Inc., 1945, ch. IV.

16955

Stabilization of the Thromboplastic Lipid by Hydroquinone.

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The instability of the thromboplastic lipid has been recognized for many years. McLean[1] demonstrated the progressive loss of thromboplastic activity of cephalin and, by concomitantly measuring its degree of unsaturation, came to the conclusion that there was direct relation between activity of cephalin and its degree of unsaturation. Similarly, Hanzlik and Weidenthal[2] showed that cephalin loses activity in 2 months.

In previous studies on the thromboplastic lipid[3] we were faced with the problem of assaying preparations over long periods of time. An effort was made therefore to protect the active agent from autoxidation using various known antioxidants. Studies were carried out with thiourea, ascorbic acid, acetone bisulfite, and hydroquinone. Of these agents, hydroquinone was by far the most effective. Deutsch *et al.*[4] had previously found that hydroquinone inhibited phospholipid oxidation catalyzed by ascorbic acid. Similarly, Rusch and Kline[5] reported the inhibition of phospholipid oxidation by polyphenolic compounds including hydroquinone. Experiments were accordingly undertaken to find the conditions necessary to prevent the autoxidation of the thromboplastic phospholipid and to determine if the thromboplastic activity would be stabilized under these conditions.

Methods. The lipid preparations used were mixtures of lecithin and cephalin isolated from beef brain and found to have considerable thromboplastic activity.[3] The mixture is a crude one and is designated by the term phospholipin for convenience.

The degree of unsaturation was determined by the Hanus iodobromine method (U. S. Pharmacopea, XI), care being taken to insure at least a 60% excess of the reagent. In the case of preparations protected against autoxidation it was found necessary first to remove the hydroquinone by washing with acetone. In all cases iodine numbers are expressed as grams of iodine reacting with 100 g of the phospholipin freed from hydroquinone.

In order to add different quantities of hydroquinone to phospholipin, a known amount of phospholipin was placed in a glass-stoppered cylinder containing 100 ml of acetone saturated with hydroquinone. Additional hydroquinone was then added to give the proportions desired and the mixture shaken well for 15 minutes. It was then filtered through a Büchner funnel and the residue dried *in vacuo*. This procedure was found to be sufficient to yield a homogenous mixture of hydroquinone and phospholipin.

The preparations were assayed for thromboplastic activity by determining the coagulation time of recalcified dog plasma with and without the addition of the thromboplastic agent. From these values the percent decrease in coagulation time produced by the agent was calculated and used as the criterion for thromboplastic activity. The technics used were the same as those described previously.[3] Two determinations were done in each case,

TABLE I.
Stability of Untreated Phospholipin.

Days	pH	Iodine No.	Activity % decrease in coag. time
0	6.49	78.4	64.2
2	6.21	77.7	64.4
5	6.30	70.4	68.4
7	5.83	59.9	61.2
10	5.30	58.3	65.1
12	5.19	55.2	57.2
15	4.93	56.8	32.4

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[1] McLean, J., *Am. J. Physiol.*, 1917, **43**, 586.

[2] Hanzlik, P. J., and Weidenthal, C. M., *J. Pharm. and Exp. Ther.*, 1920, **14**, 157.

[3] Hays, H. W., and Lein, J., *Arch. Biochem.*, 1945, **7**, 69.

[4] Deutsch, H. F., Kline, B. E., and Rusch, H. P., *J. Biol. Chem.*, 1941, **141**, 529.

[5] Rusch, H. P., and Kline, B. E., *Can. Res.*, 1941, **1**, 465.

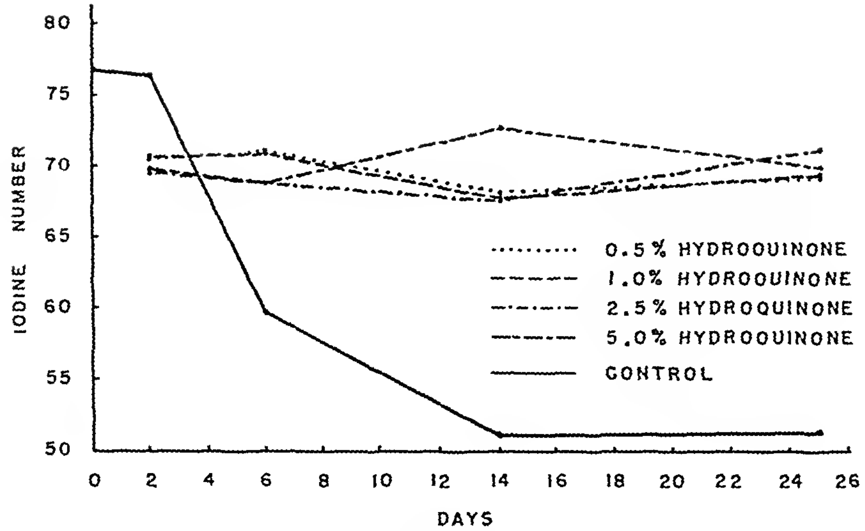

FIG. 1.

The prevention of autoxidation of phospholipin by hydroquinone in concentrations of from 0.5 to 5.0%.

and the average used, providing the deviation did not exceed 10% of the mean. In the few cases where the deviations were greater, additional determinations were carried out.

Autoxidation of Phospholipin. Phospholipin when freshly prepared is granular and has a light cream color. In the course of several days at room temperature and in diffuse light it progressively darkens, becoming orange in color after 7 days, and reddish-brown after 30 days. Concomitant with these color changes, there is a decrease in the pH of saline emulsions of phospholipin, a decrease in its iodine number and a decrease in its thromboplastic activity. Values of these changes in a typical experiment are given in Table I.

The pH values of 0.2% phospholipin emulsions were determined with a glass electrode pH meter. The pH decreased with time, the rate of decrease being greatest at about the tenth day. With a decrease in the pH value, there was a concurrent decrease in iodine number. The iodine number decreased most rapidly between the second and seventh day and tended to reach a stationary level after 12 days. The thromboplastic activity of 0.2% emulsions did not decrease markedly until the twelfth day. This long latent period was in all probability due to the fact that a 0.2% emulsion is in excess of the minimal concentration that would produce maximal activity.[3] Thus, it is evident that phospholipin is very susceptible to autoxidation which markedly alters its physiological as well as chemical characteristics.

Prevention of Autoxidation by Hydroquinone. The amount of hydroquinone added to the phospholipin was expressed as percent of the final hydroquinone-phospholipin mixture. Experiments were carried out using concentrations of hydroquinone varying from 0.5 to 5% and the iodine numbers and thromboplastic activities determined at various time intervals. Parallel determinations were carried out simultaneously on a sample of untreated phospholipin in each series.

Results of the iodine number determinations are presented graphically in Fig. 1. Complete protection against autoxidation by the amounts of hydroquinone used was obtained for 25 days while the untreated control decreased from its original iodine number of 75.7 to a final value of 51.2 in this period of time. It should also be noted that the initial iodine numbers of the hydroquinone-protected phospholipin were considerably lower than that of the control. The reason for this is not apparent but may be due to small amounts of hydroquinone that resist the washings with acetone prior to the iodine number determinations.

With this stabilization of the iodine number there occurred a stabilization of the thromboplastic activity of the preparations. After the 25 day period the percent decrease in coagulation time produced by the control was only 44 while that of the hydroquinone-protected phospholipin was 80, 79, 79, 80 for the respective 0.5, 1, 2.5 and 5% hydroquinone-phospholipin preparations.

Summary. Hydroquinone in concentrations as low as 0.5% protected a thromboplastically active phospholipid preparation from being autoxidized for a period of at least 25 days. With this protection against autoxidation there was a protection against loss of thromboplastic activity.

16956

Metabolism of Isocysteine, Labeled with Radiosulfur (S^{35}), in the Rat.

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The administration of isocysteine hydrochloride, α-thiol-β-amino-propionic acid, to rabbits results in a large excretion of "extra" sulfur in the urine.[1] Almost all of this extra sulfur is in organic combination; a large fraction of it is in the form of disulfide which is presumably isocystine, since it does not give the Sullivan[2] reaction. When isocysteine is fed to rats maintained on a diet low in proteins (6% casein), the animals' growth is not increased. The present investigation was undertaken to determine whether the sulfur of isocysteine is oxidized to sulfate by the rat.

Procedure. Isocysteine hydrochloride was prepared from α-bromo-β-alanine and labeled sodium disulfide according to the method of Schöberl and Braun.[3] Five grams of α-bromo-β-alanine hydrobromide and a solution containing 325 mg of disulfide sulfur containing S^{35} equivalent to 130,000 ± 2500 c.p.m. were used in the synthesis. The sulfur in an aliquot of the solution (in 0.1 N sodium hydroxide) of sodium sulfide which was used to prepare the disulfide for the synthesis was oxidized by alkaline fusion. A small amount of carrier was added to the resultant sulfate, and the mixture was precipitated as barium sulfate. This was washed by repeated centrifuging, first with 0.001 N hydrochloric acid and then with water, and transferred as a slurry in 70% alcohol to a tared counting cup, dried, weighed, and counted[4] using a $5/\text{mg}/\text{cm}^2$ end-window G-M tube and a scaler. Counting was continued for a sufficient time to insure an error of less than 2%. The sulfate later obtained from the specimens of biological material was precipitated, washed and counted in the same way as the sulfate from the original sulfide-sulfur solution except that no carrier was added. All counts were corrected for self-absorption and for decay.

The isocysteine hydrochloride as originally isolated was grossly impure and was purified by conversion to and recovery from its mercaptide. The final yield was 0.88 g (25%) and contained 24% of the initial radioactivity.

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* Present address: Hospital of the Rockefeller Institute for Medical Research, New York City.

† Present address: The Anderson Hospital for Cancer Research, Houston, Texas.

[1] Wingo, W. J., and Lewis, H. B., *J. Biol. Chem.*, 1946, **165**, 339.

[2] Sullivan, M. X., and Hess, W. C., *Pub. Health Rep.*, U.S.P.H.S. supplement No. 86, 1930.

[3] Schöberl, A., and Braun, H., *Ann. Chem.*, 1939, **542**, 274.

[4] Dziewiatkowski, D. D., *J. Biol. Chem.*, 1945, **161**, 723.

Its sulfur content was 19.4% (theory is 20.34%).

In each of 5 experiments, 100 mg of isocysteine hydrochloride (3627 c.p.m.) were given to young adult rats (140-168 g) from the McCollum colony at the Johns Hopkins University. In 3 cases the isocysteine hydrochloride was given by stomach tube; in 2 cases it was given by intraperitoneal injection. The rats were fasted for 24 hours before being given the compound; afterwards they were kept, still fasting, for 24 hours in individual metabolism cages. They were then killed with chloroform; and the radioactivities of the sulfur fractions of the urine, the feces, and the intestinal contents were determined. If the bladder contained urine at autopsy, this urine was added to the 24-hour specimen from the metabolism cage. Samples of the inorganic sulfate-sulfur, the total sulfate sulfur,[5] and the total sulfur[6] of the urine were isolated as barium sulfate. The feces and the intestinal contents were hydrolyzed with sodium hydroxide for 6 hours on a steam bath. An aliquot of each of these samples was acidified to Congo red paper with hydrochloric acid, filtered, heated for two hours longer, and extracted 3 times with ethyl ether; the total sulfate in the aqueous phase was then precipitated with barium chloride. Other aliquots of the alkaline hydrolysates were oxidized with sodium peroxide according to Bailey's method,[7] and the sulfate thus obtained from the total sulfur was precipitated as barium sulfate. The barium sulfate from these samples was isolated, weighed and counted according to the procedure already outlined.

Results. The data in Table I indicate that isocysteine hydrochloride is fairly well absorbed from the intestine of the rat within 24 hours, since in 2 of the 3 experiments in which the compound was given by stomach tube, 3% or less of the radioactivity of the dose given was recovered in the feces, and in 2 of the 3 experiments 5% or less was recovered from the intestinal contents. It is

TABLE I.

Excretion of Sulfur and Radioactivity in 24 Hours by Rats Following Administration of Isocysteine Hydrochloride Containing S^{35}.

	Urinary Sulfur						Fecal sulfur				Intestinal sulfur			
	Inorg. SO_4-S		Total SO_4-S		Total S		Total SO_4-S		Total S		Total SO_4-S		Total S	
Rat	mg	%‡	mg	%‡	mg	%‡	mg	%‡	mg	%‡	mg	%‡	mg	%‡
2*	5.2	9.5	5.5	9.9	21.4	66.9	0.5	0.16	20.0	2.7	1.6	0.45	4.8	2.7
3*	4.9	5.8	6.0	8.0	20.9	57.5	0.5	0.45	5.3	3.0	2.4	0.79	—	—
4*	5.2	6.6	6.0	8.7	21.4	54.2	1.3	1.1	11.8	14.6	1.1	0.53	8.8	5.0
5†	5.5	4.6	6.0	6.3	21.4	52.6	0.5	0.17	4.1	0.21	0.5	0.00	11.9	1.1
6†	6.3	5.2	6.6	7.5	24.4	52.4	0.5	0.09	4.4	0.80	0.4	0.22	8.5	1.3

* Given 100 mg isocysteine hydrochloride (3627 counts/minute) by stomach tube.
† Given 100 mg isocysteine hydrochloride (3627 counts/minute) intraperitoneally.
‡ % is noted as % of total activity administered.

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[5] Folin, O., *J. Biol. Chem.*, 1905-06, **1**, 131.

[6] Denis, W., *J. Biol. Chem.*, 1910-11, **8**, 401.

[7] Bailey, K., *Biochem. J.*, 1937, **31**, 1396.

of interest that a small but definite fraction of the radioactivity was recovered as sulfate and as organic sulfur from feces and intestinal contents of rats given the compound intraperitoneally. The activity of the feces in these experiments could have been due to contamination with urine despite precautions employed to prevent such contamination; this could not be true in the case of the intestinal contents, since these had never been in contact with urine.

It is apparent that, while a small portion of the sulfur of isocysteine hydrochloride given to rats either by stomach tube or by intraperitoneal injection may be oxidized to sulfate and excreted as such, a much larger fraction is still bound in organic combination when it is excreted. The rat, therefore, reacts similarly to the rabbit in that it does not readily oxidize the sulfur of isocysteine to sulfate.

Quantitative differences between the two species may, however, exist. The rats employed in this investigation excreted in the urine, within 24 hours, 54 to 67% of the S^{35} administered as isocysteine by stomach tube. The rabbits employed in the earlier study[1] excreted, within 24 hours, "extra" sulfur equivalent to only 29 to 37% of the dose given by stomach tube. On the other hand, the rabbits excreted "extra" sulfur equivalent to 76 to 98% of the dose when isocysteine was administered subcutaneously; rats excreted only about 52% of the S^{35} of isocysteine which had been injected intraperitoneally. The metabolic fate of the S^{35} not found in the excreta or intestinal contents of these rats cannot be ascertained from the present data, but presumably it was still contained in the tissues.

Summary.. 1. The oxidation of the sulfur of isocysteine by the rat was studied with the aid of isocysteine containing S^{35}.

2. It was found that only a small amount of S^{35} was excreted as sulfate in the urine within 24 hours after administration of the compound by stomach tube or by intraperitoneal injection. A much larger portion was excreted as organic sulfur. A very small fraction of the S^{35} was recovered in the feces and intestinal contents of the rats.

3. The results of the present study are compared with those of an earlier investigation in which rabbits served as subjects.

The authors are grateful to Dr. M. D. Kamen for the generous gift of cyclotron irradiated carbon tetrachloride from which the S^{35} employed in this investigation was isolated.

Sincere thanks are expressed to Dr. C. W. Sheppard for the loan of the scaler used in making the activity measurements.

16957

Streptomycin in the Treatment of Leptospira Carriers. Experiments with Hamsters and Dogs.*

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The carrier problem dominates the control of canine leptospirosis. Dogs remain urinary shedders for from 2 to 6 months after recovery. With the introduction of chemotherapy, the risk of creating carriers and thus spreading leptospirae is greatly increased.

Penicillin and streptomycin have been found to inhibit growth of leptospirae *in vitro* and to be effective against experimental leptospirosis in guinea pigs, mice and hamsters. Shih Lu Chang[1] observed that *Leptospira icterohaemorrhagiae* disappeared from the blood of guinea pigs 3 to 5 days after 2 daily injections of aqueous solution of penicillin in total daily doses of 800 units. A serum concentration of more than 0.2 unit per cc was maintained. However, the leptospirae persisted in the liver even when the dose was increased to 3,000 units daily and continued for 6 to 8 days after the blood tests had become negative. Wylie and Vincent[2] found that unless penicillin was given to the guinea pigs 24 hours after inoculation of *L. icterohaemorrhagiae,* the guinea pigs died. These authors stated that renal damage might have made the leptospirae inaccessible to the antibiotic.

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* Aided by a grant from the National Canine Research Foundation.

[1] Chang, S. L., *J. Clin. Invest.*, 1946, 25, 752.

Petersen and Schmidt[3] demonstrated leptospirae in the kidneys of a guinea pig 3 months after inoculation with *L. icterohaemorrhagiae* and penicillin treatment. In experiments reported by Brunner,[4] hamsters were proven renal carriers 26 days after inoculation with *L. canicola* and penicillin treatment. In discussing these results it was emphasized that although penicillin treatment may save the life of a hamster or a dog, it may favor the development of a carrier state.

Heilman and Herrell[5,6] successfully treated hamsters infected with *L. icterohaemorrhagiae* with streptomycin. Treatment was started 17 hours after inoculation, and no leptospirae were found in the livers and kidneys of the treated animals after 28 and 33 days. In hamsters infected with *L. canicola* and treated with streptomycin, Brunner failed to demonstrate the organisms in the kidneys by culture and darkfield examination.

It would appear from the foregoing that penicillin is only effective in leptospiremia, while streptomycin either prevents the leptospirae from reaching the renal tubules or is able to destroy them there. The important question of whether leptospirae multiplying in the renal tubules of chronic carriers could be reached and destroyed by streptomycin had not been answered by these studies. It is the purpose of this paper to record experiments in this direction.

Experimental work. Series 1. Forty hamsters, 4 or 5 weeks old, were each injected intraperitoneally with 0.25 cc of a virulent *Leptospira canicola* culture (120,000 organisms per cu mm). On the 60th hour after injection each hamster was given intramuscularly 500 mg of penicillin in oil and wax, and a second dose of 250 mg 12 hours later. Four hamsters died of leptospirosis after 12 to 15 days. Thirty-two days after inoculation, 9 of the 36 surviving hamsters were killed, and kidney material was examined in the dark field and cultured. All the 9 cultures were positive for leptospirae, and in 7 cases could the organisms readily be demonstrated by darkfield illumination in the fresh specimens of kidney.

Having established that the hamsters were carriers, 18 of the series were treated on 3 successive days with 5 mg of streptomycin each intramuscularly, given in oil and wax (0.1 cc volume), and 9 hamsters were left untreated as further controls. The 18 treated hamsters and the 9 untreated controls were killed on the 2nd day after the last streptomycin treatment, and kidney material was examined microscopically in the dark field and cultured. All the 18 cultures of kidney material of the treated animals were negative for leptospirae and remained sterile, whereas the cultures of the 9 controls were all positive, and in 8 the organisms could be demonstrated on darkfield examination.

In summary, then, of 36 hamsters inoculated with a virulent culture of *L. canicola* and treated with penicillin, 18 chosen at random and killed 32 to 37 days after inoculation were renal carriers, while the remaining 18 treated on 3 successive days with streptomycin were proved to have sterile kidneys 2 days after therapy had been instituted on the 33rd day after infection.

Series 2. Five dogs (about 4 months old) were infected intraperitoneally with a virulent culture of *Leptospira canicola* (about 50 million organisms per kilo body weight). Eighteen days after inoculation large numbers of leptospirae were found in the urine of all the dogs. (One dog died during the 5th week; the findings at autopsy were typical of leptospirosis.) The urine of one of the carriers was again examined 4 weeks after inoculation and found to be heavily contaminated with leptospirae. The dog (8.5 kg) was then treated with streptomycin, 700 mg on the 1st day and 500 mg on the 5 following days, given intramuscularly in oil and wax. One day after the last treatment, results of urinalysis for leptospira were negative; the same day leptospirae were found in the urine of the 3 remaining carriers. The pH values of the urine were found to fluctuate between 6 and 8; living leptospirae were not found at pH values below 7 (the dead organisms could still easily be demonstrated in the dark field). Care was taken to obtain neutral or alkaline samples from all the dogs.

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[2] Wylie, J. A. H., and Vincent, E., *J. Path. and Bact.*, 1947, **59**, 247.

[3] Petersen, B. C., and Schmidt, R. M., *Acta path. et microbiol. Scandinav.*, 1945, **22**, 462.

[4] Brunner, K. T., *California Veterinarian*, 1948, **1**, 18.

[5] Heilman, F. R., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 169.

[6] Heilman, F. R., and Herrell, W. E., *Proc. Staff Meet., Mayo Clin.*, 1944, **19**, 89.

The 3 untreated carrier dogs were then treated with streptomycin 3 days after urine had again been examined and organisms found (5 weeks after inoculation of the culture). The dose was 40 mg per kilo body weight for 4 days, given intramuscularly in oil and wax. On the 5th day after the beginning of treatment, organisms were not found in the urine (4 dogs). Darkfield examinations were made and urine was injected intraperitoneally into young hamsters.

Urine from the 4 treated dogs was again examined 11 days later and organisms were not found. The 4 dogs were then sacrificed and the kidneys examined, kidney material cultured and injected intraperitoneally into young hamsters. Results of these examinations were negative.

Series 3. Four dogs (4 months old) were given intraperitoneal injections, 2 with a virulent culture of *L. canicola* (about 120 million organisms per kilo body weight) and 2 with virulent cultures of *L. icterohaemorrhagiae* (about 30 million organisms per kilo body weight). One dog infected with *L. canicola* died on the 6th day of leptospirosis. Urine of the dogs was examined microscopically in the dark field 14 days after inoculation and found to contain large numbers of leptospirae. A second urine examination was made 9 days later, and results were again strongly positive. On the same day, streptomycin treatment of 2 dogs was started; 1 dog infected with *L. icterohaemorrhagiae* was left untreated. The daily dose used was 40 mg per kilo body weight given intramuscularly in oil and wax on 3 successive days. Two days after the last treatment urine from all 3 dogs was examined, and organisms were found only in the urine of the control dog. The 2 treated dogs were sacrificed, kidney material cultured and examined in the dark field with negative results.

Conclusions. The data here presented prove that chronic renal infections with leptospira in hamsters and dogs may be successfully cured with streptomycin. Although penicillin, as well as streptomycin, effectively influences the leptospiremia in experimental infections in guinea pigs, mice and hamsters, only streptomycin is capable of destroying the organisms found in the convoluted tubules.

Canine leptospirosis is usually treated after the parasite has already disappeared from the blood. Obviously, penicillin is of little value. Streptomycin in oil and wax in a daily dose of 40 mg per kilo given intramuscularly should be used in the treatment of acute and chronic canine leptospirosis. In fact, this antibiotic may be employed in the prevention of this destructive disease in dogs. It is recommended that the urine of all dogs intended for breeding be examined for leptospira, and when infection is found, the dogs should be treated for 3 to 5 days with streptomycin a few days before the act of breeding.

16958

Effect of Pteroylglutamic Acid on Weight and "Alkaline" Phosphatase of Kidneys of the Mouse.

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Kidney injury in guinea pigs and rabbits treated with pterolyglutamic acid (PGA) was first reported by Harned and coworkers, although PGA when given orally to rats was not harmful as judged by the normal diuretic test of Lipschitz.[1] However, these authors found a frequent precipitation of the PGA in the renal *tubuli* of the damaged kidneys. No other reports on kidney injury produced by PGA have been published so far.[2]

In the course of a toxicity study of PGA, one of us was able to find an enlargement of the kidneys in all the mice injected with PGA, even with doses far below the toxic level.[3] It has been observed that the weight of the kidneys increases markedly probably due to the precipitation of the insoluble PGA in the renal *tubuli*, which is able to induce some modifications on the enzyme content of the tissue. It was therefore of interest to investigate whether the phosphatase of the kidney was affected by injected PGA. Our first results showed that the "alkaline" phosphatase decreases even when small doses of PGA are administered subcutaneously (0.5 mg/kg). The increase of weight seems not to be due to an apparent retention of water but probably to a lowering of the excreted solids through the urine. In the present paper, the changes of the weight and of the "alkaline" phosphatase content of the kidneys after administration of low and high doses of PGA will be reported.

Material and methods. Male white mice of a homogeneous stock were used throughout this study. The animals were fasted 16 hours before autopsy and killed by exsanguination. The kidneys were immediately removed, weighed and homogenized in a mortar with distilled water. The kidneys homogenate was diluted with 40 ml of distilled water per g of tissue, according to Kochakian and Fox.[4] The samples were filtered to remove connective tissue and a final 10-fold dilution was made. King & Armstrong's method using disodium phenylphosphate as a substrate was used with slight modifications.[5] Blank determinations were run simultaneously, the incubation period being omitted. The results were expressed in units of "alkaline" phosphatase activity, one unit being equivalent to 1 mg of phenol liberated by hydrolysis. The disodium phenylphosphate solution was prepared each 2 days and stored in the ice box. In our experience older solutions give higher blanks and erratic values.

The age of the mice being a factor influencing the phosphatase content of the kidney, as showed by Kochakian and Fox, mice were selected having the same limit of age and weight and submitted to a standard diet. Adult mice weighing 20 to 22 g (65 to 90 days old) were used. Normal kidney weight and "alkaline" phosphatase values from 54 well fed mice are summarized in Table I.

Different groups of mice were injected subcutaneously with graded doses of PGA and animals killed 24 hours after the injection. The LD_{50} for this drug given intraperitoneally was found to be 600 mg/kg.[1] In our experience the dose of 375 mg/kg killed 50% of the animals when injected subcutaneously. Therefore, we used doses far below this limit to avoid acute intoxication. No death occurred in all the mice studied except for

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[1] Harned, B. K., Cunningham, R. W., Smith, H. D., and Clark, M. C., *New York Acad. Sci.*, 1946, **48**, 289.

[2] Jukes, T. H., and Stokstad, E. L. R., *Physiol. Rev.*, 1948, **28**, 51.

[3] Villela, G. G., *Arch. Biochem.*, 1947, **15**, 157.

[4] Kochakian, C. D., and Fox, P., *J. Biol. Chem.*, 1944, **153**, 669.

[5] King, E. J., Micro-Analysis in Medical Biochemistry, London, 1946, J. and A. Churchill, Ltd., p. 57.

TABLE I.
Average Values for Normal Mice and Mice Injected with PGA.

No. of mice	PGA mg/kg	Wt of kidneys, mg	σ	S.E.	Alkaline phosphatase in units					
					Total		S.E.	per g		S.E.
31	0.5-5.0	292	14.3	3.97	104	21.5	5.96	367	75.9	2.10
57	25-500	416	11.2	1.67	61.5	17.3	2.58	146	14.5	2.16
54	Controls	269	29.4	13.36	127	21.3	9.68	469	19.4	8.81

$$\sigma = \text{standard deviation} = \sqrt{\frac{\Sigma(x-m)^2}{n-1}} \qquad \text{S.E.} = \text{standard error} = \frac{\sigma}{\sqrt{n}}$$

those few injected with the dose up to 375 mg/kg.

Since a single dose of 0.5 mg/kg has only a slight effect, we tried repeated doses to know if a cumulative effect could be obtained. Five mice weighing 20-21 g were injected daily with 0.5 mg/kg until a total amount of 5 mg/kg was attained. The average weight of the kidneys was 331 mg and the "alkaline" phosphatase values were respectively 100 and 312 units for the total kidneys and per g of tissue. These results show that repeated doses of PGA produce a cumulative effect probably due to the slow rate of PGA excretion through the kidneys. The increase of weight of the kidneys is not related to the water content since the average mean values for the normal and injected mice were not significant. In one experiment 20 normal mice were used and 20 others were injected with 0.5 mg/kg to 25 mg/kg; the results for the first group showed 76.3 ± 0.3% of water and for the second group 75.4 ± 0.8%.

The inspection of the Table I demonstrates the effect of PGA on the weight and "alkaline" phosphatase of the kidneys. The difference between the means of the injected and non-injected animals is higher than twice the standard error of the mean for all groups analysed. These results are therefore statistically significant.[6] Even if the group injected with small doses is considered a significant value is obtained.

Summary. PGA when administered subcutaneously to normal mice in a single injection, produces after 24 hr an effective increase of the total weight and a decrease of the "alkaline" phosphatase of the kidneys. Normal values based on 54 mice are reported and compared with those obtained from 88 mice injected with graded doses of PGA (0.5 mg/kg to 500 mg/kg of body weight). The "alkaline" phosphatase was determined by a slight modification of the colorimetric method of King and Armstrong using disodium phenylphosphate as a substrate. The retention and precipitation of PGA in the renal *tubuli*, could be responsible for the increased weight and decreased phosphatase activity of the kidneys. Further experiments to determine the mechanism of action of PGA on kidney phosphatase are in progress and will be reported in the near future.

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[6] Fisher, R. A., Statistical Methods for Research Workers, London, 1934, Oliver and Boyd, p. 112.

16959

Benzene-Inactivated Rabies Vaccine.

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As a part of a program aimed at purification of rabies vaccine, an earlier report[1] was made on the benzene and ether extraction of ultraviolet inactivated rabies vaccine.

The technic described in our earlier paper involved the extraction of infected, dry brain tissue containing virus inactivated by ultraviolet light. Irradiation was used for inactivation because it was known to yield vaccines of high antigenic potency, and because cold benzene and ether did not inactivate all of the rabies virus. The present paper describes a method for inactivation of rabies virus by benzene during the course of extraction of lipids with that solvent.

Materials and Methods. The procedure of inactivating rabies virus by benzene differs only slightly from the method described for extraction of brain lipids.[1] A 20% infected brain suspension in distilled water is rapidly dried from a frozen state. To the dry powder is added sufficient benzene to equal 2 times the original volume. The suspension is vigorously agitated to break up clumps of tissue, poured into a screw-top bottle, and placed in a water bath at the desired temperature. After the appropriate period of time (see below) the benzene is removed by filtration through a sintered glass filter of "M" porosity by suction. The suction is then turned off and clean benzene is added to the residue on the filter. Resuspension is accomplished by stirring with a sterile applicator stick, and again suction is applied to remove the benzene. It has been established that the volume of benzene used for the first extraction is more than sufficient to dissolve the soluble lipids in the brain tissue. The first extraction is done, therefore, to dissolve lipids. The second extraction, of short duration, is done to flush out the benzene which contains lipids.

Following the extractions with benzene, the residue on the filter is resuspended in one volume of ether. The ether is permitted to filter without suction. A second resuspension in ether is done and this time the ether is removed by suction. The purpose of the ether extraction is twofold: to remove lipids—especially phosphatids which may not be soluble in benzene, and to flush out benzene not removed by filtration.

The small amount of ether which remains is removed by placing the entire filter in a vacuum chamber for a period of about 30 minutes. The dry powder is then added to a volume of distilled water equivalent to the original volume. Mixing and hydration are accomplished by churning in a Waring blender (a drop of caprylic alcohol is used to reduce the foam thus formed).

Antigenic potency of the vaccines is determined by the mouse protection test of Habel and Wright.[2]

The Temperature and Time Necessary for Inactivation of Rabies Virus (P M strain 980). The principle of using higher temperatures for the inactivation of rabies virus in benzene was derived from the early experiments in which the virus was treated with benzene at 5°C. It was observed at that temperature that the titer of live virus was markedly decreased, but the antigenic potency was not adversely affected. Subsequently, the vaccine was extracted at 37°C and no decrease in antigenicity was observed, but the titer of live virus was more markedly diminished.

It was subsequently found that 24 hours at 37°C would usually result in inactivation of the virus but occasionally viable virus could

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[1] Wright, John T., Bell, J. Frederick, and Habel, Karl, *Science*, 1948, **108**, 118.

[2] Habel, Karl, and Wright, John T., *Public Health Rep.*, 1948, **63**, 44.

TABLE I.

Lot No.	Animal species and source of vaccine	Benzene inactivated Temp., °C	Benzene inactivated Time, hr	Benzene inactivated LD_{50} prot., × 1000	Ultraviolet inactivated, LD_{50} prot., × 1000
1	Mouse	37	144	>417	289
2	”	37	72	>289	246
3	Rabbit	37	96	1.2	1.2
4	”	37	96	0.25	0.23
5	Mouse	56	8	35	35
6	Rabbit	56	8	1.7	2.2
7	”	56	9½	10	2.8
8	”	56	12	1.2	1.4

be detected. An obvious reason for this variability in inactivation seemed to be that some clumps of dry tissue were not broken up in benzene when simply shaken in that solvent. Subsequent extractions for 24 hours at 37°C using more finely divided tissues consistently yielded non-viable antigens.

For routine inactivation of the virus, however, it is desirable to submit the tissues to more than the bare minimum of exposure usually necessary for such inactivation, as long as antigenicity is not lost by this over-exposure. There has been no indication that over-exposure to benzene at 37°C results in any loss of antigenicity, and there is direct evidence that exposure for as long as 5 days at that temperature is not harmful.

Because benzene at 37°C was not inimical to the vaccine, even on prolonged exposure, it seemed probable that a higher temperature might be used for extraction. The following data compare the potencies of vaccines prepared from one lot of rabies infected brain emulsion exposed to temperatures of 37°, 45°, and 56°C for 72 hours during benzene extraction. All 3 vaccines were negative when tested for viable virus.

37°C for 72 hrs	LD_{50} protection	>195,000
45°C ” ” ”	” ”	>195,000
56°C ” ” ”	” ”	11,400

It is apparent that 56°C for 72 hours caused a marked reduction in antigenicity, but did not destroy it completely.

The next step was to determine the minimum time necessary for rendering all the virus non-viable at 56°C. It was found that virus thus exposed for 9 hours was inactivated, but that at 5 hours sufficient live virus remained to infect one out of 6 mice when a 10% whole brain suspension was injected intracerebrally. One hour at 56°C was sufficient to effect a ten-fold drop in titer.

The effect of prolonged heating at 56°C in benzene upon the antigenicity of the virus is exemplified below:

56°C 12 hr	LD_{50} protection	11,300
” 24 ”	” ”	27,600
” 48 ”	” ”	<4
” 70 ”	” ”	<4

The vaccine extracted for 24 hours appears to have greater antigenicity than that heated for only 12 hours, but this difference is not significant. However, at some time between 24 hours and 48 hours the potency of the vaccine is almost completely destroyed.

From these data a procedure has been derived for the routine preparation of vaccines which accomplishes the two primary objectives of high antigenicity and non-viability. In ordinary use the virus has been exposed to benzene at 56°C for 12 hours. This treatment has resulted in uniformly high potency of various lots of vaccine, and has consistently yielded non-infectious vaccines.

The Lipid Content of Benzene-Treated Vaccines. In the previous report it was stated that benzene-ether extraction at 5°C effected a removal of 41% of the total dry weight of brain tissue. This statement was in error since subsequent studies have established by extraction of brain tissue with hot alcohol and ether that the lipid content of rabbit brain and of mouse brain are 50% and 40% respectively, of the total dry weight. After extraction with benzene and ether the residual lipids constitute about 25% of the dry weight of both rabbit and mouse brain tissue.

Comparative Antigenicities of Benzene and Ultraviolet Inactivated Vaccines. The relative antigenicities of benzene inactivated as compared with ultraviolet light inactivated vaccines, each made from the same lot of virus, are listed in Table I.

In most instances, when there were differences, the benzene inactivated vaccine showed the higher potency.

In addition to these comparisons showing benzene inactivated vaccines were as good as the ultraviolet inactivated vaccines, more than 20 other lots have been inactivated with benzene alone and have consistently produced good vaccines.

Discussion and Summary. The use of benzene-ether extraction as a preliminary step in eventual purification of rabies vaccines has been made a more practical procedure by the demonstration that inactivation of the virus by heating in benzene results in a highly potent vaccine. Thus the necessity for preliminary ultraviolet irradiation has been eliminated.

16960

Rabies Vaccine Freed of the Factor Causing Allergic Encephalitis.

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The preliminary phases of a program for the purification of rabies vaccine have been reported in earlier papers. These reports dealt with the problems of removal of lipids from brain tissue vaccines,[1] and the inactivation of rabies virus by heating the dried tissue in a lipid solvent (benzene).[2] Earlier attempts at the purification of rabies antigen by the usual chemical and physical fractionation procedures had met with failure. Brain tissue is rich in lipids and it was thought that these might have the effect of stabilizing the suspension of other constituents including virus, thus inhibiting sharp separation. The first step in separation of antigen, therefore, was the removal of lipids. For this purpose benzene and ether were used, and it was found that vaccines thus treated did not lose antigenicity. A corollary finding was the observation that the rabies virus could be rendered non-infectious by heating during the course of benzene extraction, thus obviating the necessity for preliminary inactivation by ultraviolet light or other agents.

Postvaccinal (rabies vaccine) encephalomyelitis in man, and its apparent counterpart, experimental allergic encephalitis in lower animals, are conditions which occur when brain tissue is injected parenterally.[3-7] The encephalomyelitis may be manifest as a paralysis of the Landry type. Pathognomonic lesions are found in the central nervous system.

A consideration of the etiology of this postvaccinal encephalomyelitis had led to the hope that removal of the lipid components of brain tissue might also eliminate the material which was responsible for the paralysis.[8] However, it was found that benzene-ether extracted vaccine was essentially unaltered in its capability of producing paralysis in guinea pigs.

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[1] Wright, J. T., Bell, J. F., and Habel, K., *Science*, 1948, **108**, 118.

[2] Habel, K., Wright, J. T., and Bell, J. F., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 455.

[3] Freund, J., Stern, E. R., and Pisani, T. M., *J. Immun.*, 1947, **57**, 179.

[4] Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

[5] Kopeloff, L. M., and Kopeloff, N., *J. Immun.*, 1947, **57**, 229.

[6] Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

[7] Wolf, A., Kabat, E. A., and Bezer, A. E., *J. Neuropath. and Exp. Neurol.*, 1947, **6**, 333.

[8] Alvord, E. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 459.

The present report will describe the further treatment of rabies vaccines which has resulted in antigens of high potency, yet freed of the factor which causes allergic encephalitis in experimental animals.

Materials and Methods. In each experiment, one lot of vaccine, or a pool of several lots was used, and comparisons of potency were made between various fractions of the one lot or pool. In every instance the various fractions were brought back to the volume of the original vaccine suspension before being compared. In some of the early experiments the potencies of the vaccines used were so great that no end-point of protection in mice was obtained, and therefore no comparisons of the potencies could be made. In later experiments, an attempt was made to select lots of vaccine which would protect mice when challenged with 100 to 10,000 LD_{50}, which figures are within the usual range of the end points of the mouse protection test. Both mouse and rabbit brain vaccines were used. In all of the tests for the factor causing allergic encephalitis, only rabbit brain vaccines were employed.

In order to elicit the signs and lesions of allergic encephalitis, guinea pigs were injected in the nuchal area with vaccines made up in mineral oil containing killed *M. tuberculosis* and Arlacel-A according to the method of Freund.[3,9] One injection of one ml of the adjuvant mixture was made subcutaneously in the nuchal area of the guinea pigs, and early signs of encephalitis usually ensued about 12 to 14 days later. The animals were observed for about 7 weeks after injection. Characteristic lesions of allergic encephalomyelitis sometimes occur in guinea pigs which remain asymptomatic. Therefore, random samplings, or in some cases, all of the guinea pigs in groups without signs of encephalomyelitis were killed at the termination of the experiments, and the brains and cords were examined for microscopic lesions.*

Calcium Acetate as a Precipitant for Virus Antigen. In the course of some experiments on the elution of live rabies virus from tissue it was observed that calcium acetate greatly reduced the titer of virus in the supernatant liquor of a centrifuged suspension of rabies brain. That this effect was not caused by killing the virus was proved by resuspending the sediment (in molar sodium chloride solution) and again centrifuging the suspension. The titer of virus thus obtained in this second supernatant fluid was as high as was ordinarily expected in the original. Further investigation revealed that the virus treated with calcium acetate, and apparently precipitated by it, could not be resuspended in water. Non-viable virus antigen likewise was precipitated by calcium acetate and remained insoluble in water. This precipitated antigen retained its antigenic potency.

In the first experiment rabies-infected brain was homogenized in a 1% solution of calcium acetate. The mixture was centrifuged and the titer of virus in the supernatant fluid was found to be low. Further experiments revealed that concentrations as low as M/100 (0.158%) of calcium acetate could be used with maximal effect. In these experiments the brain tissue vaccines were suspended in various concentrations of calcium acetate and filtered. The filtrates were then used as antigens in the mouse potency test.

It is evident here that calcium acetate in concentrations of M/100 or greater prevented passage of antigen through the filter.

The insolubility in water of virus antigen precipitated by calcium acetate is shown in Table II. In these experiments, vaccines which had been extracted by benzene and ether were suspended in water ("original"). Sufficient calcium acetate solution was added to the suspensions to make a final concentration of M/10 and the suspensions were centrifuged. (The supernatant fluid is referred to as "calcium acetate wash"). After decanting the supernatant fluid, the sediment was resuspended in a volume of distilled water equal to the first supernatant fluid, and the mixture was again centrifuged. (The supernatant

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[9] Freund, J., Thomson, K. J., *et al.*, *J. Immun.*, 1948, **60**, 383.

* The pathological studies were done by Dr. James Peers.

TABLE I.
Antigenic Potency of Filtrates of Vaccines in Various Concentrations of Calcium Acetate in H_2O, Expressed in LD_{50} Protection.

Exp. No.	M/10	M/100	M/1000	No Ca Ac
A 168	<22	<22	>220,000	220,000
A 179	<36	<36	1,500	20,000

TABLE II.
Antigenic Potency of Vaccine Fractions in LD_{50} Protection.

Exp. No.	Original	Cal. acetate wash	Water wash	Residue
A 187	69,000	420*	<42	11,000
A 188	2,900	<29	<29	360
A 195	1,200	<17	<17	1,100

* M/100 calcium acetate was used instead of M/10 and insufficient time for reaction was allowed.

TABLE III.
Comparative Potencies of Original and Washed Vaccines (LD_{50} Protection).

Lot	Original	Washed
A 142	>381,000	>381,000
A 152	470	1,200
A 158	> 66,100	17,800
A 163	> 32,400	> 32,400
A 177	>513,000	16,300

fluid is labeled "Water Wash"). The sediment was resuspended in distilled water to the original volume ("Residue").

The comparative antigenic potencies of other benzene-ether extracted vaccines and their washed calcium acetate residues are presented in Table III.

It has been observed that the most effective way of adding calcium acetate to the vaccine is by the addition of a solution of the chemical to a suspension of the vaccine. (Other methods such as the addition of the calcium acetate to the vaccine before drying and extracting with benzene and ether, adding the dried extracted powder to a solution of calcium acetate, or adding the chemical to a vaccine resuspended after extraction were tried. The last procedure was satisfactory but less consistent in results.)

It will be noted in the experiments recorded in Table II that the potencies of the residues are less than those of the control vaccines. While variations of less than three-fold may be within the limits of error of the mouse potency test, some of the apparent loss of potency seems to be the result of the poor dispersion of the precipitated antigen when resuspended. This belief is substantiated by the decrease in antigenicity caused by the addition of calcium acetate directly to vaccine as compared to the same whole emulsion vaccine without calcium acetate. (See Table IV.)

This effect could be interpreted as an actual diminution of antigen, but the lack of toxicity of calcium acetate to live virus, and the relative constancy of the effect without regard to time of exposure or concentration of the salt have led us to believe that the difference is caused by poorer dispersion of the precipitated antigen.

The Nitrogen and Calcium Contents of Washed Vaccine. Repeated analyses of vaccine before and after washing with M/10 calcium acetate followed by water have revealed that the first wash (*i.e.*, with calcium acetate) removes about 45% of the total nitrogen, and the subsequent washing with water removes about 5%. The residual washed vaccine then contains about 50% of the total nitrogen of the original vaccine. A small portion of the calcium added to the vaccine is retained by the insoluble tissue components. Most of this is removed by

TABLE IV.
Effect of M/10 Calcium Acetate on Antigenic Potency* of Crude Rabies Vaccine.

Vaccine	Without calcium acetate	With calcium acetate
A 183	>355,000	60,300
A 188 K	10,300	2,900
A 188 L	2,900	1,100

* Potency is expressed in LD_{50} protection.

TABLE V.
Pathogenic and Antigenic Potencies (LD_{50} Protection) of Crude and Washed Vaccines.

			Allergic encephalitis	Antigenic potency
A188	Unextracted	Original vacine	9/10	1,100
		Cal. acetate wash	1/8	<29
		Washed vaccine	2/9	130
	Extracted	Original vaccine	4/10	2,900
		Cal. acetate wash	9/10	<29
		Washed vaccine	0/9 *	360
A195	Unextracted	Original vaccine	5/8	1,400
		Cal. acetate wash	5/10	<17
		Washed vaccine	7/10	1,200
	Extracted	Original vaccine	7/10	1,200
		Cal. acetate wash	8/10	<17
		Washed vaccine	0/10†	1,000

The denominator indicates the number of animals injected. The numerator indicates those specifically affected.

* Five animals were killed and the brains and cords were submitted to histological study. None showed lesions of allergic encephalitis.

† All of the animals in this series were killed and the brains and cords were found free of the lesions of allergic encephalitis.

washing with water. The final calcium content of the washed vaccine (10% vaccine) is then about 0.005%.

Extraction of the Factor Causing Allergic Encephalomyelitis. When it was discovered that much water soluble material could be removed from the vaccine without loss of antigen by the use of the calcium acetate it seemed worth while to test the various fractions thus obtained for the encephalitic effect. Consequently, a portion of a lot of vaccine, after washing with calcium acetate, was then treated with molar saline in an attempt to elute antigen from the sediment. The original lot of vaccine, the calcium acetate wash liquor, the molar saline wash liquor, and the final residue were each mixed with adjuvant and injected into guinea pigs. The results were as follows:

Original vaccine (Lot A169)	2 of 8 *guinea pigs paralyzed*
Calcium acetate wash	7 of 10 ” ” ”
Saline wash	4 of 10 ” ” ”
Residue	None of 9 ” ” ”

It was obvious then that one washing with calcium acetate was insufficient to remove all the factor. However, it was equally evident that the factor was soluble in the calcium acetate solution. That guinea pigs injected with the second wash liquor were also affected could readily be explained by the fact that not all of the calcium acetate solution could be removed from the residue by drainage. That this encephalitic factor can be completely removed by a second washing with water is demonstrated by the experiments reported in Table V.

In these experiments the effects of the washing procedures on antigenicity as well as the encephalitic factor of the vaccines were tested. Both benzene-ether extracted and unextracted irradiated vaccines were subjected to the calcium acetate treatment. The extracted and unextracted vaccines of each lot were suspended in M/10 calcium acetate solution and samples were removed for testing ("Original"). The remainder was centrifuged and the supernatant fluid ("calcium acetate wash") replaced by an equal volume of distilled water. The sediment was resuspended in water and again centrifuged. After decanting and measuring the supernatant fluid, the sediment was suspended in the same quantity of M/1 saline. Antigenic potency as recorded in Table V in the row marked "washed vaccine" was determined for this resuspended sediment. However, for the detection of the factor causing allergic encephalitis in the washed vaccines only the liquid phase of the mixture was used.

The data indicate that the factor which causes encephalomyelitis was present in the original vaccines, both extracted and irra-

TABLE VI.
Absence of the Encephalitic Factor from Washed Vaccines (Whole Washed Sediments).

Vaccines		Encephalitis	Antigenicity (LD_{50} protection)
A 200	Original vaccine	8/10	4,080
	Washed vaccine	0/10	1,000
A 201	Original vaccine	3/5	340
	Washed vaccine	0/5	120

diated, and that it was soluble in the calcium acetate washes. After washing once with calcium acetate solution, and once with distilled water, no more of the factor could be demonstrated in the vaccines which had previously been extracted with benzene and ether. However, the method does not appear to be effective with vaccine which has not been previously extracted.

Further evidence that the factor has been completely removed from washed vaccine is presented in Table VI where the whole washed sediment was tested.

Discussion. By a process of extraction with calcium acetate solution, rabies vaccine may be freed of the factor which causes allergic encephalitis. Absence of the encephalitic factor from the washed vaccine was first indicated by the fact that it was not present in the liquid phase of the final washed vaccine. Further proof was obtained by injecting the whole washed vaccine into guinea pigs without producing paralysis.

In the experiments recorded here calcium acetate has been used because it has been found to give satisfactory results. No doubt other salts of calcium, and salts of other alkaline earth elements may be substituted. In fact, calcium chloride has been found to react very similarly.

Whether the allergic encephalomyelitis as produced in guinea pigs is identical with that occasionally seen in man following rabies vaccination is not known, but the signs and lesions, together with the common factor of development of the syndromes following the injection of brain tissue suggest that the conditions are actually similar.

The results of our experiments have been reported here because of their possible application to the production of safe rabies vaccine for human use. It is presumed that washed rabies vaccines which no longer produce allergic encephalitis in experimental animals will likewise not cause post vaccinal paralysis in man. Further purification of vaccine for human use is desirable, and the problem is being studied.

Summary. A method has been presented for the removal from rabies vaccine of the factor which causes allergic encephalomyelitis. This factor is not removed by extraction with benzene and ether, but preliminary treatment of the vaccine with these solvents facilitates separation by subsequent treatment. The presence of calcium acetate prevents the loss of antigen when the vaccine is washed. It does not prevent removal of the encephalitic factor which appears to be water soluble. About one-half of the total nitrogen of the vaccine is removed by this washing process. The technic may be briefly summarized as follows:

1. A suspension of infected brain in water is dried from the frozen state.
2. The dried brain is extracted with benzene followed by ether (Live virus may be killed in this stage by heating in benzene).
3. After removal of the ether the dried brain is suspended in distilled water.
4. Sufficient solution of calcium acetate is added to make final concentration of M/10 calcium acetate, and the suspension is permitted to stand in the cold for an hour or two.
5. The calcium acetate solution is removed by centrifugation or filtration and the sediment is resuspended in distilled water to the original volume with agitation (clumps of sediment must be broken up).
6. The distilled water is removed by centrifugation or filtration.
7. The sediment is resuspended in distilled water or saline and homogenized. This is the washed vaccine.

TABLE V.

Pathogenic and Antigenic Potencies (LD_{50} Protection) of Crude and Washed Vaccines.

			Allergic encephalitis	Antigenic potency
A188	Unextracted	Original vacine	9/10	1,100
		Cal. acetate wash	1/8	<29
		Washed vaccine	2/9	130
	Extracted	Original vaccine	4/10	2,900
		Cal. acetate wash	9/10	<29
		Washed vaccine	0/9 *	360
A195	Unextracted	Original vaccine	5/8	1,400
		Cal. acetate wash	5/10	<17
		Washed vaccine	7/10	1,200
	Extracted	Original vaccine	7/10	1,200
		Cal. acetate wash	8/10	<17
		Washed vaccine	0/10†	1,000

The denominator indicates the number of animals injected. The numerator indicates those specifically affected.

* Five animals were killed and the brains and cords were submitted to histological study. None showed lesions of allergic encephalitis.

† All of the animals in this series were killed and the brains and cords were found free of the lesions of allergic encephalitis.

washing with water. The final calcium content of the washed vaccine (10% vaccine) is then about 0.005%.

Extraction of the Factor Causing Allergic Encephalomyelitis. When it was discovered that much water soluble material could be removed from the vaccine without loss of antigen by the use of the calcium acetate it seemed worth while to test the various fractions thus obtained for the encephalitic effect. Consequently, a portion of a lot of vaccine, after washing with calcium acetate, was then treated with molar saline in an attempt to elute antigen from the sediment. The original lot of vaccine, the calcium acetate wash liquor, the molar saline wash liquor, and the final residue were each mixed with adjuvant and injected into guinea pigs. The results were as follows:

Original vaccine (Lot A169)	2 of 8	guinea	pigs	paralyzed
Calcium acetate wash	7 of 10	,,	,,	,,
Saline wash	4 of 10	,,	,,	,,
Residue	None of 9	,,	,,	,,

It was obvious then that one washing with calcium acetate was insufficient to remove all the factor. However, it was equally evident that the factor was soluble in the calcium acetate solution. That guinea pigs injected with the second wash liquor were also affected could readily be explained by the fact that not all of the calcium acetate solution could be removed from the residue by drainage. That this encephalitic factor can be completely removed by a second washing with water is demonstrated by the experiments reported in Table V.

In these experiments the effects of the washing procedures on antigenicity as well as the encephalitic factor of the vaccines were tested. Both benzene-ether extracted and unextracted irradiated vaccines were subjected to the calcium acetate treatment. The extracted and unextracted vaccines of each lot were suspended in M/10 calcium acetate solution and samples were removed for testing ("Original"). The remainder was centrifuged and the supernatant fluid ("calcium acetate wash") replaced by an equal volume of distilled water. The sediment was resuspended in water and again centrifuged. After decanting and measuring the supernatant fluid, the sediment was suspended in the same quantity of M/1 saline. Antigenic potency as recorded in Table V in the row marked "washed vaccine" was determined for this resuspended sediment. However, for the detection of the factor causing allergic encephalitis in the washed vaccines only the liquid phase of the mixture was used.

The data indicate that the factor which causes encephalomyelitis was present in the original vaccines, both extracted and irra-

TABLE I.
Growth Rate of *M. tuberculosis var. hominis* (Strain 7800) in Percentages in the Presence of PAS and Salicylate. (Uninhibited growth = 100%.)

	PAS μM								
Salicylate, μM	50	25	12.5	6.25	3.12	1.56	0.76	0.38	0.00
200	0	18	29	32	40	40	46	57	65
80	0	0	0	9	46	61	65	68	94
40	0	0	0	0	8	35	50	70	96
20	0	0	0	0	0	11	53	72	100
0	0	0	0	0	0	0	26	67	100

ing. This complete inhibition was not antagonized by either PAB or pantothenic acid. A slight growth (about 8 to 12%) was, however, found if a certain concentration (about 2 μM of PAS), which in itself alone is fully bacteriostatic, was added to the tube.

Similar observations were made with strains 5110 and K3. The effect of salicylate on strain K5, however, was found to be very slight.

The tuberculostatic effect of PAS was not antagonized by compounds related to salicylic acid. No antagonism was found when the Na salts of benzoic, phthalic, o-aminobenzoic, o-phenolsulfonic, o-chlorobenzoic, o-nitrobenzoic, dithiosalicylic, o-sulfobenzoic, p- and m-oxybenzoic, 2, 4-dioxybenzoic and 2-oxy-4-aminosulfobenzoic acids were tested in the range of concentrations between 40 and 200 μM. m-Aminophenol and pantothenic acid were also found to be ineffective.

Discussion. The action of salicylate on bacteria is a variable one. The growth of some non-acid fast bacteria is prevented by the drug at high concentrations by inhibiting the synthesis of pantothenic acid in the cells.[5] The non-pathogenic acid fast bacteria are capable of oxidizing salicylic acid,[6] and the oxygen uptake of resting virulent tubercle bacilli is increased in the presence of this drug;[7] however, it is not metabolized[8] by the cells. Fitzgerald and Bernheim[9] concluded from their experiments that acid fast bacteria produce an adaptive enzyme in the presence of salicylate, and that the formation of this enzyme is inhibited by streptomycin. On the basis of these observations it can be assumed that both non-acid fast bacteria requiring no exogenous pantothenic acid for growth and various acid fast bacteria either contain or form adaptively an enzyme with specific affinity to salicylic acid. However, the synthesis of pantothenic acid in tubercle bacilli is not inhibited by this drug. The nature of this enzyme (or enzymes) occurring in a wide variety of bacteria is entirely obscure at present.

Salicylate antagonizes the tuberculostatic effect of PAS at concentrations so high as to be toxic by themselves. As found previously, salicylate has a twofold action on bacteria: in addition to a specific effect at high dilutions, the drug will damage the cells in high concentrations by its protein-denaturing effect.[5,10] This latter action of the drug renders the study of antagonism of the two drugs investigated more difficult; however, it seems that it is a competitive one.

Summary. The bacteriostatic effect of p-aminosalicylic acid is antagonized by high concentrations of salicylic acid. This effect of salicylate is highly specific and not shared by related compounds. Pantothenic acid does not inhibit the tuberculostatic effect of salicylate.

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[5] Iványovics, G., *Z. f. physiol. Chem.*, 1942, **276**, 33.

[6] Bernheim, F., *J. Biol. Chem.*, 1943, **143**, 383.

[7] Bernheim, F., *Science*, 1940, **92**, 204.

[8] Fitzgerald, R. J., and Bernheim, F., *J. Bact.*, 1947, **54**, 671.

[9] Fitzgerald, R. J., and Bernheim, F., *J. Bact.*, 1948, **55**, 765.

[10] Iványovics, G., Csábi, J., and Diczfalusy, E., *Hungarica Acta Physiol.*, 1948. **1**, 171.

16961

Antagonism Between Effects of P-Aminosalicylic Acid and Salicylic Acid on Growth of *M. tuberculosis.*

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It has been reported[1,2] that the bacteriostatic action of PAS is not antagonized by small amounts of salicylic acid. Lehmann[3] found in some of his experiments that a combination of PAS and salicylic acid gave a moderate inhibition of oxygen uptake of tubercle bacilli, although each of these acids, added separately, increased the rate of oxygen uptake. It was expected that an antagonism between salicylic acid and P-aminosalicylic acid (PAS) can also be demonstrated in cultures of tubercle bacilli if the experimental conditions are chosen correctly.

Methods. Cultures were grown in the liquid medium described by Dubos. It contained 0.05% of Tween 80 and 0.5% of bovine albumin fraction. A freshly prepared solution of the Na salt of PAS was added to the medium and serial dilutions by a factor of 2 were made. The tubes were inoculated with 0.0001 mg (dry weight) of tubercle bacilli grown in Dubos medium, and the total volume of the cultures was made up to 5 ml with dist. water. After 16 days of incubation at 37°C, 0.5 ml of phosphate buffer pH 7.0 containing 2% formaldehyde and 0.1% Tween 80 was added to each tube and the amount of tubercle bacilli was determined by turbidimetric readings in a Leitz universal colorimeter. Using a control culture as reference, the growth rate of bacilli in the presence of antiseptics was expressed in percentages of the full growth. Each dilution of drug or combination of drugs was tested in duplicates, and turbidimetric readings were averaged.

Four strains of *M. tuberculosis var. hominis* were used in these experiments. Two of them were isolated from sputa recently (K3 and K5); the others (Nos. 7800 and 5100) have been maintained for several years in our collection.

Results. Using strain 7800, the effect of PAS alone and in combination with sodium salicylate was studied simultaneously.

The striking antagonism between Na salicylate and PAS is shown in Table I. Although a concentration of 200 μM of Na salicylate alone markedly inhibited the growth of tubercle bacilli, this amount antagonized the effect of about 30 minimal inhibiting concentrations of PAS. The tuberculostatic action of PAS is antagonized only by a considerable concentration of Na salicylate, and the molar ratio of PAS to Na salicylate in the range of minimal inhibition varied between 0.08 to 0.25, *i.e.*, one mole of PAS was antagonized by 4 to 12 moles of Na salicylate in this experiment.

In the presence of small concentrations of Na salicylate antagonism gradually diminishes, and at the highest dilution of drug tested (20 μM) only a slight effect was seen.

As the tuberculostatic action of PAS is antagonized by p-aminobenzoic acid (PAB),[4] we determined the molar ratio of PAS to this metabolite under similar experimental conditions. It was found that one mole of PAB abolishes the effect of about 4 moles of PAS. On comparing these values it is seen that the antagonistic effect of PAB is 16 to 50 times greater than that of salicylate.

In the presence of salicylate at a concentration of 620 μM the growth of strain 7800 was found to be zero on turbidimetric read-

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[1] Youmans, G. P., Raleigh, G. W., and Youmans, A. S., *J. Bact.*, 1947, **54**, 409.

[2] Fitzgerald, R. J., and Bernheim, F., *Am. Rev. Tuberc.*, 1948, **58**, 210.

[3] Lehmann, J., *Svenska Läkartidn.*, 1946, **43**, 2029.

[4] Goodacre, C. L., Mitchell, B. W., and Seymour, D. E., *Quart. J. Pharm. and Pharmacol.*, 1948, **21**, 301.

TABLE I.
Sensitivity of Gram Negative Bacilli to Aureomycin *in vitro*.

Strain	No. of bacteria, × 1000	Bactericidal titer, in μg/ml	Bacteriostatic titer, in μg/ml
Ps. aeruginosa 1	20	>200	>200
” ” 2	18	100	100
” ” 3	20	50	50
” ” 4	15	12.5	12.5
” ” 5	25	3.2	3.2
” ” 6	25	0.8	0.8
” ” 7	18	0.4	0.4
B. proteus 1	2.5	50	50
” ” 2	1.5	25	12.5
” ” 3	3	6.25	6.25
” ” 4	4	0.05	0.05
” ” 5	1.5	0.025	<0.012
” ” 6	1.5	<0.012	<0.012
A. aerogenes 1	2	>200	>200
” ” 2	2.5	100	100
” ” 3	2	3.2	3.2
” ” 4	2.5	0.4	0.4
” ” 5	2	0.05	0.05
E. coli 1	4	25	6.25
” ” 2	5	6.25	3.2
” ” 3	6	1.0	1.0
” ” 4	5	0.1	0.1

mycin, penicillin, and the sulfonamides.

Four patients had univalent and 6 polyvalent infections. All patients had pyuria and the usual clinical signs and symptoms.

Aureomycin was given intramuscularly to 9 patients. The dose usually was 80 mg every 8 hours for 5-6 days, but acutely ill patients received an initial intravenous dose of 300 mg in 500 cc of saline. The tenth patient received aureomycin orally, 4 g the first day, 3 g the second day and 2 g daily thereafter, in divided doses. The duration of treatment in all cases was based on the clinical and bacteriological response. No toxic reactions were encountered. The intramuscular injections were painful. The pain was partly or wholly alleviated by dissolving the drug in 1% procaine. Table II lists the clinical diagnosis, the bacteriological findings before and after aureomycin therapy, *in vitro* sensitivity tests of the bacteria involved to various antibiotics, and the therapy given prior to aureomycin.

The results were evaluated on a clinical and a bacteriological basis. The clinical symptoms cleared up in all patients. In 3 of the 4 patients with univalent infections and in 5 of the 6 patients with polyvalent infections the urine became sterile and remained so during the 7-10 days after the drug was discontinued. In one case (No. 3), the urine was not completely sterilized, but the plate count showed a marked reduction in the number of colonies of the responsible bacillus (*A. aerogenes*). This strain, tested *in vitro* for sensitivity, was resistant to sulfathiazole, sulfadiazine, sulfamethazine, penicillin, and streptomycin. The bactericidal titer with aureomycin was higher than 200 μg. In the second case, (No. 6), one of the 2 infecting organisms, *Ps. aeruginosa*, was eliminated, while the plate count of the other, *A. aerogenes*, was markedly reduced. This latter strain was also resistant *in vitro* to the 3 sulfonamides, penicillin and streptomycin. The bactericidal titer with aureomycin was again higher than 200 μg.

Of the 20 strains of bacteria found in the 10 patients, all strains of *E. coli* (4), of *Ps. aeruginosa* (4), of *B. proteus* (2), of *Strep. viridans* (2), of *Strep. fecalis* (1), and 5 of the 7 strains of *A. aerogenes* were cleared from the urine. The two strains of *A. aerogenes* which persisted showed a high resis-

16962

Aureomycin in Urinary Infections Due to Gram Negative Organisms.*

ALEXANDER M. RUTENBURG AND FRITZ B. SCHWEINBURG.
(Introduced by J. Fine.)
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Aureomycin hydrochloride ("Duomycin")† has been reported to be a non-toxic antibiotic effective against a variety of gram-positive and gram-negative bacteria, rickettsiae and several of the larger viruses.[1-4]

This report deals with the results of aureomycin therapy in urinary tract infections due to gram-negative bacilli in 10 patients, 9 of whom did not respond to sulfonamides, penicillin or streptomycin.

In vitro studies. Data on the *in vitro* sensitivity to aureomycin of bacteria commonly encountered in urinary infections were obtained prior to the clinical study.

Serial dilutions of aureomycin in sterile broth (pH 6.8) were prepared in concentrations ranging from 200 - 0.0125 µg/ml. To 0.9 ml of each dilution 0.1 ml of a diluted 24 hour growth, containing 1500-25,000 organisms of the strain to be tested, was added. A control tube containing the same number of bacteria in 1.0 ml of sterile broth was also prepared. The tubes were incubated for 24 hours and then examined for bacterial growth. Ten strains each of *Pseudomonas aeruginosa* (*B. pyocyaneus*), *Bacillus proteus vulgaris*, *Aerobacter aerogenes*, and *Escherichia coli* were tested for aureomycin sensitivity in this manner. All strains were isolated from infected urines and had undergone only 1-3 passages. The results, listed in Table I, indicate the lowest bactericidal and bacteriostatic titers of aureomycin. The bacteriocidal titer was taken to be the lowest concentration which completely inhibited macroscopically visible growth. The bacteriostatic titer was taken to be the lowest concentration which caused less growth than in the control tube.

The results, only half of which are listed, show that different strains of a given species of gram-negative bacteria have a wide range of sensitivity. Thus, the bactericidal titer of *Ps. aeruginosa* varies from >200 µg to 0.4 µg; of *B. proteus* from 50 µg to <0.0125 µg; of *A. aerogenes* from >200 µg to 0.05 µg; and of *E. coli* from 25 µg to 0.1 µg. *Ps. aeruginosa* and *B. proteus* have been reported[1,4] to require very high concentrations of aureomycin (100-250 µg/ml). We found this to be true of some strains, but not of others. The bacteriostatic titers for a given strain of *Ps. aeruginosa*, *B. proteus* and *A. aerogenes* were identical in most instances with the bactericidal titer; but for *E. coli* a marked difference in the two titers was the rule. The bactericidal titer of most strains of all bacteria tested varied from 1.0 µg to 0.0125 µg, indicating a high degree of sensitivity. The bactericidal titer was always the same for a given strain tested with different lots of aureomycin.

Clinical Observations. Ten consecutive patients with a variety of urinary tract infections were treated with aureomycin. Prior therapy with sulfonamides in 7, with penicillin in 6, and with streptomycin in 2 had failed to yield any therapeutic effect. In all instances the infecting bacteria had been tested and found to be resistant *in vitro* to strepto-

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* Aided by a grant from the Patrons of Research, Beth Israel Hospital.

† An antibiotic isolated from *Streptomyces aureofaciens* and supplied by Lederle Laboratories Division, American Cyanamid Co.

[1] Bryer, M. S., Schoenbach, E. B., Chandler, C. A., Bliss, E. A., and Long, P. H., *J.A.M.A.*, 1948, **138**, 117.

[2] Wright, L. T., Sanders, M., Logan, M. A., Prigot, A., and Hill, L. M., *J.A.M.A.*, 1948, **138**, 408.

[3] Braley, A. E., and Sanders, M., *J.A.M.A.*, 1948, **138**, 424.

[4] Paine, T. F., Jr., Collins, H. S., and Finland, M., *J. Bact.*, 1948, **56**, 489.

[5] Cooke, C., *J.A.M.A.*, 1948, **138**, 885.

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TABLE II.
The Data of Ten Patients with Urinary Infections Successfully Treated with Aureomycin.

Case No.	Diagnosis	Urine cultures before aureomycin	Growth inhibiting concentration *in vitro* of aureomycin, μg/ml	Urine cultures after aureomycin	Sensitivity tests *in vitro* with ather antibiotics*	Chemotherapy prior to aureomycin	Aureomycin Daily Dose (mg)	Aureomycin Days
1	Chronic pyelonephritis, renal insufficiency	*A. aerogenes* *E. coli* *Strep. viridans*	6.25 3.2 0.8	Sterile	All 3 strains resistant to SD, ST, SMT, P and S.	None	240	6
2	Chronic pyelonephritis, ectopic kidney	*E. coli* *B. proteus*	0.05 12.5	,,	Both strains resistant to P, S, SD, and SMT. *Proteus* also to ST. *Coli* moderately sensitive to ST	SD	240	5
3	Chronic pyelonephritis	*A. aerogenes* heavy growth	>200	*A. aerogenes* 1-4 colonies	Strain resistant to P, S, SD, ST, SMT	Penicillin Streptomycin	80	8
4	Pyelonephritis following prostatectomy	*B. pyocyaneus* *Strep. fecalis*	50	Sterile	*B. pyocyaneus* resistant to SD, ST, SMT, P, and S.	Penicillin SD	120	6
5	Same	*E. coli* *A. aerogenes* *B. pyocyaneus* *B. proteus* *Strep. viridans*	25 200 0.25 0.25	,,	*Coli*, *aerogenes*, *pyocyaneus*, and *proteus* resistant to P, SD, ST, SMT. *Coli* and *aerogenes* slightly sensitive to S. *Proteus* and *pyocyaneus* resistant to S.	Penicillin SD SMT	160	10
6	Same	*A. aerogenes* *B. pyocyaneus*	>200 1.6	*A. aerogenes* 3-6 colonies	Both strains resistant to P, S, SD, SMT. *B. pyocyaneus* resistant to ST. *A. aerogenes* sensitive to ST.	SD Penicillin	160–240	15
7	Chronic cystitis	*E. coli* *A. aerogenes*	0.2 0.2	Sterile	Both strains resistant to P, S, SD, SMT. Moderately sensitive to ST.	Penicillin Streptomycin	60	12
8	Acute pyelonephritis and cystitis due to renal calculi	*A. aerogenes* heavy growth	0.2	,,	Strain resistant to P, S, SD, SMT, ST.	SD Penicillin	240	5
9	Acute pyelonephritis and cystitis due to renal calculi	*B. pyocyaneus* heavy growth	12.5	,,	*B. pyocyaneus* resistant to P, S, SD, SMT, ST.	SD Penicillin	240 2000†	4 3
10	Acute postoperative cystitis	*A. aerogenes* heavy growth	0.0125	,,	*A. aerogenes* resistant to P, S, SD, ST, SMT.	SMT	2000–4000†	6

* SD—Sulfadiazine. ST—Sulfathiazole. SMT—Sulfamethazine. S—Streptomycin. P— Penicillin.
† Oral administration.

tance to the drug *in vitro,* but were nevertheless reduced in number. Strains of *Ps. aeruginosa* and *B. proteus* which are sensitive *in vitro* to aureomycin can be eliminated from urine infected with these organisms. In all patients who were cured treatment was continued for 2 or 3 days after the urine was first found to be sterile, because in one patient not listed, on whom the drug was discontinued as soon as the urine became sterile, recurrence took place within 2 days.

Summary. 1. Aureomycin is highly effective against many strains of gram-negative bacilli *in vitro,* including many which were found to be resistant to sulfonamides, penicillin, and streptomycin. The sensitivity of various strains of a given species, however, varies greatly.

2. Clinical trial in 10 patients with urinary infections which were refractory to previous treatment with other antibiotics, resulted in a clinical cure of all ten patients. Bacteriologically eight patients were cured, and 2 were markedly improved.

16963

Effects of a Quaternary Amine[*] Capable of Blocking Functions of the Autonomic Nervous System.

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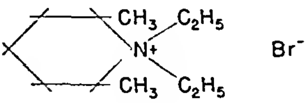
From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

Much interest has been directed in recent years to functions of the autonomic nervous system, and to effects of drugs which interfere with activation of adrenergic or cholinergic end organs or with cholinergic synaptic transmission in autonomic ganglia. Acheson and Moe[1] and Lyons[2] have reported effects of the tetraethylammonium ion (TEABr) in the experimental animal and in man, and have shown that it blocks cholinergic transmission at autonomic ganglia and does not prevent activation of adrenergic end organs.

2, 6 dimethyl diethyl piperidinium bromide (SC 1950), a quaternary amine (Fig. 1), has been synthesized and studied by I. C. Winter and associates.[3] They found that this drug was 7 times as potent as TEABr in blocking transmission through the superior cervical ganglion of the cat and 5 times as potent as TEABr in producing block of transmission through the ganglion of the pelvic nerve which supplies the urinary bladder of the dog. It had little spasmolytic action on excised intestine, but in anesthetized and unanesthetized dogs following a preliminary contraction it generally reduced intestinal tone and contractility. Intravenous injection in anesthetized dogs produced a decrease of blood pressure. They also noted that large doses (20-25 mg/kg) in unanesthetized animals produced a flaccid paralysis and respiratory death resembling that caused by curare.

Experiments were undertaken to investigate effects of this drug upon the circula-



2,6 dimethyl diethyl piperidinium bromide

FIG. 1.

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[*] Drug number SC 1950 furnished by the Research Laboratories of G. D. Searle and Co. Aided by a grant from G. D. Searle and Co.

[†] U. S. Public Health Service Research Fellow in Surgery.

[1] Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, **87**, 220.

[2] Lyons, R. H., Moe, G. K., Neligh, R. B., Hoobler, S. W., Campbell, K. N., Berry, R. L., and Rennick, B. R., *Am. J. Med. Sc.*, 1947, **213**, 315.

[3] Personal communication from Dr. I. C. Winter of the Research Laboratories of G. D. Searle and Company.

tory system and gastrointestinal tract of dogs and of man.

I. Action of SC 1950 in the dog. A. Effect on the circulatory system. Pulse rate, blood pressure, vasomotor reflexes, and response to epinephrine and neostigmine were determined in seven dogs weighing 9-16 kg. Each was anesthetized using intravenous chloralose, 0.1 g/kg. A tracheal cannula was inserted, the carotid arteries were isolated, and the vagi were divided. A left lumbar sympathectomy was performed transperitoneally. The right femoral artery was cannulated proximally and distally. The left was ligated proximally and cannulated distally. This permitted recording of 3 blood pressures using a mercury manometer connected to the proximal cannula for systemic blood pressure and 2 others connected to the distal segments for back pressure from the 2 femoral arteries. Respirations were measured by a pneumograph connected to a recording tambour.

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(1) *Effect on blood pressure and pulse.* Initial mean systolic blood pressure of anesthetized animals varied from 80 to 120 mm Hg. With 3 exceptions initial and subsequent doses of SC 1950 in amounts varying from 0.05 mg/kg to 20 mg/kg reduced pressure. The 3 exceptions occurred when blood pressure had been reduced by previous injections. Transient increases of pressure then occurred ranging from 6 to 34 mm Hg and lasting less than 2½ minutes. Other than for these three exceptions SC 1950 promptly reduced pressure. With doses ranging from 0.05 to 1.0 mg/kg reductions of blood pressure were moderate and transient, lasting 2 to 5 minutes. As dose was increased depression of blood pressure became more pronounced and duration of the period of depression lengthened. Following doses of 5 to 20 mg/kg blood pressure was reduced to ½ or 1/3 the level before injection and depression persisted for 25 to 50 minutes. A representative effect is shown in Fig. 2. After long periods of anesthesia and after several doses of SC 1950, blood pressure gradually declined and did not return to its initial level between injections.

Effect on cardiac rate also varied with amount of drug injected. Small doses (0.02 to 1.0 mg/kg) produced no change. Larger doses (5 to 20 mg/kg) caused slowing of heart rate which persisted as long as did depression of blood pressure. Before larger doses of SC 1950 pulse rates varied from 104 to 208, averaging 159. Following 5 to 20 mg/kg, pulse rates fell to from 56 to 132, averaging 93.

(2) *Effect of SC 1950 on vascular reflexes.* In the 4 animals receiving larger amounts of SC 1950 the rise of blood pressure normally occurring in response to occlusion of the carotid arteries was uniformly prevented. This response was completely abolished in each of 10 test periods following injections of 5 mg/kg or more. (Fig. 2) Stimulation of the proximal end of the divided right vagus normally produced marked increase of blood pressure and cessation of respiration (Fig. 2).

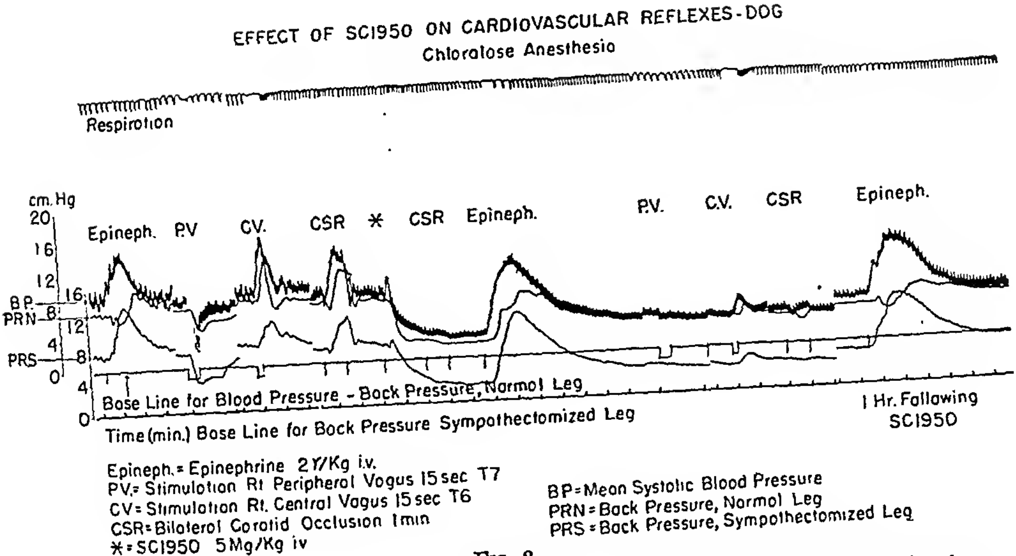

FIG. 2.

Systemic blood pressure and back pressures in normal and sympathectomized extremities (see text) are shown in response to epinephrine, peripheral and central vagal stimulation, and bilateral carotid occlusion, before and after 5.0 mg/kg SC 1950.

After 5 mg or more of SC 1950 the increase of blood pressure was blocked during all tests on 3 dogs. In the fourth dog this response was reduced but not abolished following 5 mg/kg and again following 10 mg/kg 2 hours later. Normally stimulation of the distal end of the divided right vagus effected cardiac slowing or arrest and fall of blood pressure. In 3 animals after SC 1950, 5 to 20 mg/kg, this response was completely blocked, heart rate continuing unchanged during vagal stimulation. In a fourth 5 mg/kg reduced but did not prevent cardiac slowing. Ten mg/kg 90 minutes later, however, prevented slowing. Anoxia produced an elevation of blood pressure of 20 mm Hg before the drug in only one of these 4 animals. After the drug this response did not occur. It is of interest that following removal of the gas an overshoot of blood pressure occurred once before and once after the drug. In the 3 dogs remaining small amounts of SC 1950 (0.02 to 1.0 mg/kg) did not block reflexes.

Blood pressure measured in the distal ends of the divided femoral arteries, representing "back pressure" or, indirectly, peripheral resistance, was lower in the sympathectomized extremity and responded slowly or passively in this limb during reflexes before the drug. Following SC 1950 peripheral or back pressure in the normal extremity decreased to equal that in the sympathectomized limb, and responses to cardiovascular reflexes were equally slow and passive.

(3) *Effect on response of blood pressure to intravenous epinephrine.* Epinephrine, 2 to 4 γ/kg, injected intravenously normally produced blood pressure increases ranging from 30 to 56 mm Hg following which the pressure returned to preinjection levels in two minutes or less. Following administration of SC 1950, 5 to 10 mg/kg, and during the associated reduction of systemic blood pressure, pressor responses to equal doses of epinephrine increased, reaching 46 to 168 mm Hg, a response 2 to 4 times as great as that before SC 1950 (Fig. 2). The duration of the pressor response also increased, ranging from 3.5 to 7.0 minutes following SC 1950 as compared with 2 minutes or less prior to the drug. Even after systemic blood pressure had returned to normal levels a half hour or more after injections of SC 1950, increased and prolonged responses to epinephrine occurred. Before SC 1950 back pressure of the normal leg decreased immediately following intravenous epinephrine and then increased. Initial decreases did not occur in the sympa-

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less than one minute to slightly less than 8 minutes. Average time of initial emptying was 4.1 minutes in 11 animals. Time required for complete emptying of barium from the stomach varied markedly in a few normal animals but in most the emptying times were similar. Average amount of emptying at ½ hour was 30%, at 1 hour 46%, and at two hours 84%. Gastric emptying was complete at 3 hours in 8 of 10 dogs and at 4 hours in 9 of 10. Of 9 dogs in which observations could be made with accuracy, the first of the barium had traveled through the ileum and reached the colon at 2 hours in 3 dogs, and at 3 hours in 6 dogs.

On another day these control animals were given SC 1950 intravenously and barium sulfate by stomach tube as before 10 minutes following drug. Doses of 0.4 to 1.0 mg/kg were used in 5 animals. These small doses caused an acceleration of initial gastric emptying in 4, all beginning to empty in less than 2 minutes, and no change in the fifth. In the first 4 dogs subsequent emptying of the stomach was also accelerated, averaging 68% in ½ hour, 88% in 1 hour and 95% in 2 hours. The fifth dog had a normal emptying rate. The first barium reached the colon in 2 hours in 3, and 3 hours in 2.

Larger doses, 5 and 10 mg/kg, were then administered to 6 dogs. In each of these animals there was a definite delay of time of initial emptying and of total emptying. In 4 cases transit time through the small intestine was also prolonged. The time of initial emptying varied from 10 minutes to more than an hour, average 29 minutes. At ½ hour 4 animals showed no emptying, one 1% emptying, and one 20% emptying. By 1 hour average emptying was 17%, one animal still having complete gastric retention and 2 others having passed only traces of barium into the small intestine. At 2 hours emptying averaged 60%, at 3 hours 71%, and at 4 hours 82%. In the 4 dogs receiving 10 mg/kg SC 1950, barium traversed the ileum and reached the colon only after 4 to 6 hours. In the 2 receiving 5 mg/kg, barium appeared in the colon at 2 and 3 hours.

Fluoroscopy following the smaller doses (0.4 to 1.0 mg/kg) of SC 1950 revealed little change in gastric motility from the control animals. Following larger doses peristalsis was markedly decreased in 5, but appeared normal in one although initial and total emptying was delayed.

II. *Action of SC 1950 in Man.* Doses of SC 1950 varying from 0.5 to 2.0 mg/kg or 31 to 150 mg per patient were administered intravenously and of 2.0 mg/kg or 100 to 192 mg per patient intramuscularly to 53 patients without evident ill effects and without subsequent abnormality evident in blood counts or urinalysis. Immediately after intravenous injection patients had reddening of the skin and experienced a feeling of warmth. Most had a sense of general relaxation and some became slightly drowsy. Within a few minutes dryness of mucous membranes, dilatation of pupils, and loss of accommodation were noted. Occasionally ptosis of the upper eyelids and engorgement of conjunctival blood vessels appeared. Most of these changes persisted 30 to 60 minutes after intravenous injection. Following intramuscular administration these effects of the drug were noted in approximately 15 minutes and persisted 2 to 3 hours. One patient had pain from a duodenal ulcer at the time the drug was given and experienced relief for 1½ hours following 1.0 mg/kg intravenously. Another had right upper quadrant pain persisting 3 months following cholecystectomy and experienced relief for 110 minutes following 1.5 mg/kg. A burning pain of the foot in another, evidently associated with diabetic neuropathy, was repeatedly relieved for several hours following injections of 1.5 mg/kg. Several patients experiencing nausea before SC 1950 were relieved immediately after its injection. Oral administration of 2 to 15 mg/kg (100 to 900 mg total dose) produced only slight and inconsistent effects.

A. *Effect on Cardiovascular System of Man.* With the exception of one patient who had slight pressor responses with repeated doses of SC 1950, 0.5 to 1.5 mg/kg, lowering of blood pressure was consistently produced by the drug. The decrease following intravenous injection in normotensive patients thectomized leg. After the drug such initial decreases no longer occurred in the normal leg. The maximum increase of back pressure or "peripheral resistance" occurring after epinephrine was not as great in the normal extremity as in the sympathectomized extremity either before or after SC 1950.

(4) *Reversal of SC 1950 by neostigmine.* In 4 instances 0.5 to 1.0 mg neostigmine methylsulfate was administered intravenously after doses of 5 to 20 mg/kg SC 1950 had produced the above described reduction of blood pressure and block of reflexes. In each instance blood pressure rose promptly to levels near those before SC 1950, and the vascular reflexes again became active after neostigmine.

(5) *Effect on response to increased intracranial pressure.* Increase of intracranial pressure was effected by injection of saline into the skull through a trocar in 4 dogs anesthetized with chloralose. Intracranial pressure was kept greater than mean systolic blood pressure until death occurred. In normal animals[4] the terminal or agonal increase of blood pressure usually exceeds 200 mm Hg. This hypertensive response was not reduced in 3 dogs given 5 to 15 mg/kg of SC 1950, an amount adequate to block the pressor response to occlusion of the carotid arteries. The response to increased intracranial pressure was partially blocked in the fourth, receiving 15 mg/kg. The increases of blood pressure during increase of intracranial pressure after SC 1950 were respectively 58 to 218, 100 to 280, 54 to 204, and in the fourth dog 40 to 98 mm Hg.

(6) *Effect on pulse and blood pressure of unanesthetized dogs.* SC 1950, 2 to 15 mg/kg, was given intravenously to trained dogs whose blood pressure had been measured frequently by puncture of a femoral artery using a needle connected to a mercury manometer. Some of these dogs had been made hypertensive 3 months to one year previously by excision of the carotid sinuses and division of the cardio-aortic depressor nerves.[5] Others were normal animals without hypertension. Mean systolic blood pressure in the normal dogs ranged from 112 to 140 mm Hg (average 126) before the drug. In 12 tests on these normal dogs 2 to 15 mg/kg SC 1950 was injected intravenously. There were moderate increases of blood pressure in 11, blood pressures after the drug ranging between 130 and 162, the average being 147 mm Hg. Two of these dogs developed an initial rise of less than 20 mm Hg followed by a fall of pressure to below normal. One dog only had an immediate depressor response, blood pressure falling from 126 to 104 mm Hg. The moderate rise of blood pressure occurring after the drug lasted an average time of 36 minutes. All normal unanesthetized dogs developed increases of pulse rate following SC 1950, pulse rates before drug being 72 to 116 per minute, average 88, and after drug being 112 to 168, average 142 per minute.

In 11 tests on dogs made hypertensive by excision of the carotid sinuses and division of the depressor nerves, blood pressure before SC 1950 ranged from 198 to 264, average 223 mm Hg. Intravenous injection of 2 to 10 mg/kg SC 1950 consistently caused a decrease of blood pressure to levels ranging from 76 to 154, average 127. This reduction of blood pressure lasted 30 to 230 minutes, averaging 115 minutes. Pulse rates of these dogs ranged from 104 to 248, average 180 before the drug. Afterward the rate ranged from 84 to 144, average 114.

B. *Effect on the Gastrointestinal Tract.* Fluoroscopic and roentgenographic studies of the gastrointestinal tracts of 11 normal dogs were undertaken following administration of 30 g of barium sulfate in a 100 cc aqueous suspension by stomach tube. Fluoroscopy was performed immediately after administration of the barium observing gastric activity and timing the interval between administration of barium and initial emptying of the stomach. Roentgenograms were obtained 30 and 60 minutes following ingestion of barium and at hourly intervals thereafter until 6 hours had elapsed. Before the drug gastric peristalsis was active in all animals. Emptying of barium into the duodenum began in from

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[4] Grimson, K. S., Wilson, H., and Phemister, D. B., *Ann. Surg.*, 1937, 106, 801.

[5] Grimson, K. S., *Arch. Surg.*, 1941, 43, 284.

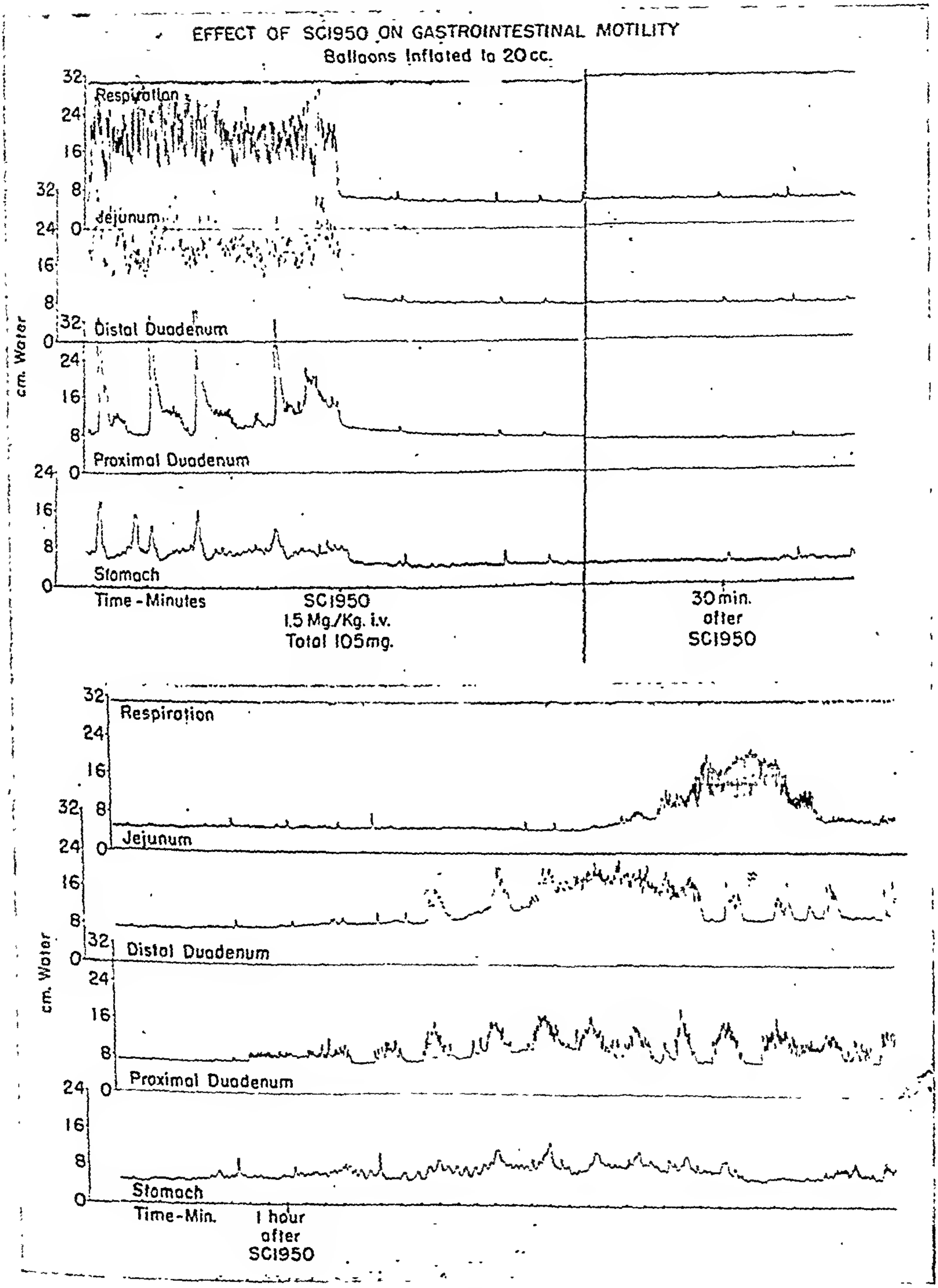

FIG. 3.

Activity of the stomach and upper small intestine as measured by 4 balloons 8 inches apart. SC1950 reduces tone and stops contractions for a period of one hour. Recovery occurs first in the more proximal segments.

in the supine position was from an average of 125/77 before the drug to an average of 94/62 afterward. In hypertensive patients in the supine position the reduction was from an average of 190/130 before the drug to 114/80 afterward. The greatest fall of blood pressure occurred within two minutes following intravenous injection, following which pressures gradually rose to their former levels in from 10 to 120 minutes, usually about ½ hour. With standing, decrease of blood pressure (postural hypotension) was more pronounced. This postural hypotension occurred immediately after the drug and could be demonstrated a half hour or longer following return of supine blood pressure readings to levels equal to those before the drug.

Pressor responses normally occurring with immersion of the hand in ice water for 60 seconds and with breath holding were tested before and after SC 1950 in 12 patients. In each of the 12 pressor responses were obtained with both stimuli before the drug. After 1.0 to 1.5 mg/kg pressor responses to cold were prevented or reversed in 9 cases, reduced in 2, and still active in one. Pressor responses to breath holding were blocked or reversed in 8 patients, and reduced in 4.

The temperature gradients of lower extremities were tested at a constant room temperature of 25° C before and after 1.0 to 2.0 mg/kg SC 1950 in 12 patients. Skin temperatures were obtained with a Cambridge thermocouple. Before the drug toes were several degrees colder than the umbilicus. Afterward in 9 patients the gradient was completely abolished, warmth of toes and feet equaling that at the umbilicus. In one patient with arteriosclerotic occlusive vascular disease gradient was reduced but not abolished. Another with marked arteriosclerotic arterial insufficiency had no change of gradient. The final patient had no change of gradient. She weighed only 44 kg and had only 66 mg total dose of SC 1950.

As a rule cadiac rate was not markedly affected by SC 1950. Most patients had a slight tachycardia during the hypotensive action of the drug. A few patients with tachycardia before SC 1950 had moderate cardiac slowing following the drug.

B. *Effect on Human Gastrointestinal Activity.* Balloon studies of activity of the stomach and small intestine were performed in 20 patients. Gastric motility was studied using a full-sized condom fastened over the tip of a No. 14F Levine tube. Balloon tension or pressure at 300 cc was first recorded on the kymograph. The balloon was then deflated and passed with the tube into the stomach, placing the tip approximately 5 inches past the cardia under fluoroscopic guidance. The Levine tube was connected to a bromoform manometer and the balloon then reinflated to 300 cc by 50 cc increments of air. Rate of respirations was recorded by a pneumograph attached to the chest of the patient and connected to a tambour. In 2 patients studies of activity of the small intestine were made with a small balloon passed into the intestine on a Miller Abbot tube and inflated to 20 cc. In 3 more detailed studies were carried out employing the 4 lumen tube method described by Chapman[6] with the proximal balloon in the stomach or upper duodenum and 3 other balloons 8 inches apart distributed in the duodenum and jejunum.

Intravenous administration of 1.0 to 1.5 mg/kg SC 1950 consistently caused an immediate decrease of resting intragastric pressure or "tone" and cessation of forceful fluctuation of pressure or "peristalsis" of the stomach and the small intestine. The "tone" of the stomach began returning 25 to 40 minutes following SC 1950 and after 60 to 80 minutes tone was often slightly greater than before the drug. Gastric peristaltic activity was entirely absent for 30 minutes or more in all cases, and an average time of 58 minutes elapsed before any return of contractility was noted. Complete recovery of gastric activity was never regained in less than one hour, and usually 1½ hours were required before contractions became relatively normal.

Observations of activity of the small intestine closely paralleled those described for the stomach, reduction of tone and cessation of contractions occurring after the drug. When the 4 lumen tube was used it was noted that increased tone and contractions reap-

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[6] Chapman, W. P., Stanbury, J. B., and Jones, C. M., *J. Clin. Invest.*, 1948, 27, 34.

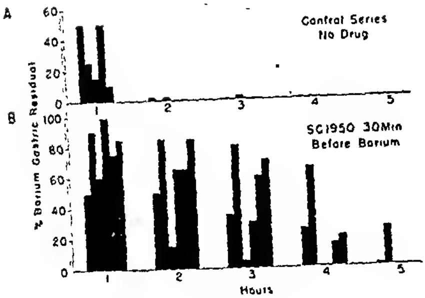

FIG. 4.

Roentgenographic study of the human gastrointestinal tract. Gastric emptying before and after 2 mg/kg SC 1950 IM.

or cecum. Barium had reached the cecum at 1, 2, 3, 3, and 4 hours respectively.

On another day SC 1950 was given to these same patients in amounts of 2 mg/kg intramuscularly, 30 minutes before ingestion of barium. Following SC 1950 and immediately after giving barium no peristaltic activity was noted by fluoroscopy in any patient. Only one demonstrated slight emptying immediately after barium. At one hour only one patient had as much as 50% emptying, and average emptying was 25%. At 2 hours there was a 40% average emptying with variations from 15% to 85% (Fig. 4B) Also at 2 hours the most distal barium was in the duodenum or jejunum in 5 patients and in the upper ileum in one. At 4 hours 2 of 6 had 100% gastric emptying and at 6 hours 4 of 6 had 100% emptying. By 4 hours barium had reached the cecum in all control patients but in only one of 6 patients after SC 1950.

C. *Effect on Gastric Secretions.* Preliminary studies have been performed on 4 patients testing effect of SC 1950 on volume and acidity of gastric secretions. Continuous gastric aspiration was employed, and samples were taken every 15 minutes. These patients all had duodenal ulcers and secreted large volumes of highly acid gastric juice. Following SC 1950 (1.5 mg/kg) intravenously, volume and acidity of gastric secretions was markedly diminished for an hour or more. Free acid was present in all specimens prior to SC 1950. Afterward there was no free acid in 2 or more specimens from 3 patients. In the fourth the total acid secreted was reduced from an average of 0.51 m eq during each 15 minute period to an average of 0.09 m eq during each 15 minute period. Although free acid never disappeared, this reduction of acidity following SC 1950 persisted 1¼ hours.

Conclusion. A new quaternary amine, 2,6, dimethyl, diethyl piperidinium bromide, described as a ganglionic blocking agent,[4] has an inhibitory or blocking action upon functions mediated by the sympathetic and parasympathetic divisions of the autonomic nervous system. In dog and in man it reduces blood pressure and prevents several vasomotor reflexes, but does not prevent the vasopressor action of epinephrine. It causes a reduction of tone of the stomach and small intestine and abolishes their motor activity, reduces gastric acidity, and causes pupillary dilatation, loss of accommodation, and dryness of mucous membranes. Urecholine causes a reversal of effect on gastric motility. Neostigmine apparently causes a reversal of all effects of the drug.

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Specificity of Differential Sheep Cell Agglutination Test in Rheumatoid Arthritis.*

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Rose and his associates[1] recently described a sheep cell agglutination test which may prove to be helpful as an aid in the differential diagnosis between rheumatoid arthritis and other diseases with arthritic manifestations. These authors found that a 16-fold difference

TABLE I.
Effect of Urecholine and Prostigmine on Human Gastric Activity Following SC 1950 I.V. Dose 1.5 mg/kg. Gastrometric Studies. (300 cc Balloon.)

Gastric activity following SC 1950	Interval before second drug (min.)	Second drug	Total dose, No. mg	Route	Recovery time (min.) Following second drug	Recovery time (min.) Following SC 1950
0	12	Urecholine	2.5	I.M	20	32
0	12	"	2.5	"	19	31
0	13	"	4.0	"	15	28
0	5	"	5.0	"	6	11
0	7	Prostigmine	1.0	"	41	48
0	11	"	0.5	"	23	34
0	12	"	0.5	I.V.	8	20
0	10	"	0.5	"	11	21

peared first in the balloon located most proximally and then successively in the more distal balloons. Usually 6 to 8 minutes elapsed before activity appeared in successive segments 8 inches apart (Fig. 3). Four to 10 minutes after resumption of peristaltic activity the small intestine occasionally became excessively active for a period of 8 to 10 minutes. The duration of the period of reduced tone and absence of contractions following doses of 1.5 mg/kg SC 1950 was with one exception an hour or more and averaged 72 minutes. The exception occurred in a patient weighing only 40 kg, receiving a total dose of 80 mg. Total cessation of activity in this patient lasted 40 minutes. In the small intestine normal contractions appeared within a few minutes of resumption of activity.

Four subjects were given 1.5 mg/kg SC 1950 intravenously and 12 minutes later were given Urecholine 2.5 to 5 mg intramuscularly. In each patient SC 1950 produced lowering of tone and complete cessation of activity as measured by gastric balloons (Table I). After Urecholine all developed a slight increase of tone within 10 minutes and all had resumption of peristaltic activity within 20 minutes following Urecholine, or 11, 28, 31, and 32 minutes after SC 1950. As described above the average time of return of peristalsis following SC 1950 alone was 58 minutes, only one patient of 8 showing return of gastric activity 30 minutes following administration. Four other gastric motility studies were done giving neostigmine 0.5 mg 7 to 12 minutes after tone had been decreased and contractions abolished by 1.5 mg/kg of SC 1950 given intravenously. In 2 of these studies 0.5 mg neostigmine was given intravenously and in 2 intramuscularly. Motility returned 8 and 11 minutes following intravenous neostigmine or 20 and 21 minutes following SC 1950 (Table I). In one of 2 patients receiving 0.5 mg neostigmine intramuscularly, motility returned 23 minutes following neostigmine and 34 minutes following SC 1950. The other subject received two intramuscular injections of 0.5 mg neostigmine, the first 7, the last 22 minutes following 1.5 mg/kg SC 1950 intravenously. No recurrence of contractions was noted until 41 minutes following the first neostigmine or 48 minutes following the SC 1950.

Six patients with apparently normal gastrointestinal tracts had fluoroscopic and roentgenologic studies performed after ingestion of barium. Five of these had control studies in which 120 g barium sulfate suspended in 200 cc water was given orally. Fluoroscopy was done immediately and gastric activity and rate of emptying were noted. Roentgenograms were obtained at hourly intervals for 6 hours following ingestion. No abnormalities were noted. Gastric peristalsis was evident in all immediately after ingestion of barium and emptying into the duodenum occurred promptly. At one hour gastric emptying was at least 50% complete in all, average being 70% (Fig. 4A). The most distal barium was in the ileum at one hour in all. Two hours after ingestion 98%-100% of barium had passed through the stomach, and the most distal barium had advanced to the midileum, terminal ileum,

TABLE I.

Results of Differential Sheep Cell Agglutination Tests on Serum Specimens from Patients with Rheumatoid Arthritis and a Variety of Other Conditions.

Clinical diagnosis*	No. of cases	Distribution of cases according to differential agglutination titer									
		0	2	4	8	16	32	64	128	256	512
Rheumatoid arthritis	35	1	8	6	4	6	5	4			1
Rheumatoid spondylitis (Marie-Strümpell)	7		4	1	1†	1†					
Reiter's disease	1		1								
Arthralgia	1		1								
Osteo-arthritis	3	1	1		1						
Gonorrheal arthritis (?)	1		1								
Scleroderma	3		1		2						
Lupus erythematosus disseminatus	1		1								
Acute rheumatic fever	18	9	8	1							
Infectious mononucleosis	2	2									
Lymphogranuloma venereum	14		11	3							
Syphilis	17	5	10	2							
Infectious diseases without evidence of arthritis‡	18	4	9	5							
Other diseases without evidence of arthritis§	9	2	6	1							
Presumably normal persons	18	3	13	10	2						

* In all instances the clinical diagnosis was substantiated by appropriate laboratory tests when availble.

† This patient also had peripheral joint involvement.

‡ These include one case each of brucellosis, infectious hepatitis, equine encephalomyelitis (Eastern type), primary atypical pneumonia, typhus fever, pneumococcal pneumonia, and poliomyelitis, 2 cases each of psittacosis and toxoplamosis, 3 cases of lymphocytic choriomeningitis, and 4 cases of Q fever.

§ These include one case each of acute glomerulonephritis and Hodgkin's disease, 2 cases each of myelogenous leukemia, carcinoma and multiple myeloma.

or greater between agglutination titers with sensitized and unsensitized sheep erythrocytes occurred almost exclusively with sera from patients with rheumatoid arthritis. The differential agglutination titer usually reflected the clinical activity of the disease. Although the serologic properties of the sera of rheumatoid arthritis patients have been extensively studied, no test of definite diagnostic value has been devised.[2-5] It therefore seemed desirable to evaluate the differential sheep cell agglutination test.

Methods and Materials. In performing the test, the procedure described by Rose and his associates[1] was followed in detail. Tests were read immediately upon removal from the refrigerator. The end point of each titration was read as the highest serum dilution showing one-plus agglutination. The titer was recorded as the reciprocal of that serum dilution. The differential agglutination titer as presented in the accompanying tables represents the difference in titers obtained in tests conducted with unsensitized and with sensitized sheep erythrocytes and is recorded as the algebraic difference between these titers.

The test was performed with serum from 43 patients with rheumatoid arthritis including 7 patients with rheumatoid spondylitis (Marie-Strümpell) and one patient with juvenile rheumatoid arthritis (Still's disease). In all but one of these patients the disease was considered clinically active. For purposes of comparison, sera from 88 individuals with a variety of other conditions including diseases with and without evidence of arthritis were examined. Also included were serum specimens from 18 presumably normal persons.

Results. The results of the differential sheep cell agglutination test performed with serum specimens from 148 individuals are summarized in Table I. It is evident from these data that serum specimens from only 17 of the 42 patients with rheumatoid arthritis showed differential agglutination titers of 16 or greater. These were all from clinically active cases. Thus, these observations confirm, in part, those of Rose and his associates.[1] There were 24 cases of active rheumatoid arthritis and one inactive case of long duration in which a differential agglutination titer of less than 16 was obtained. In this respect these observations differ from those previously recorded.[1] Of the remaining 106 serum specimens from individuals with a variety of conditions, including 18 presumably normal persons, none showed a differential sheep cell agglutination titer greater than 8 and all except 5 had titers less than 8. The serum from only one of 18 patients with acute rheumatic fever (juvenile) showed a differential titer as great as 4. Fourteen cases of lymphogranuloma venereum which had been proved by the Frei skin test and by complement-fixation with a yolk sac antigen were included because of the reported occurrence of increased titers of sheep cell agglutinins in this disease.[6][†] None of the serum specimens from these patients showed a differential titer greater than 4.

Table II summarizes the results of the differential sheep cell agglutination test in the group of patients with rheumatoid arthritis exclusive of those with spondylitis. All but one of the patients showed evidence of active joint disease at the time specimens were obtained. In the tabulation, the cases are classified, without knowledge of the results of the agglutination test, according to the severity and duration of the illness. The following were taken into consideration when classify-

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[*] Aided in part by grants from the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y., and the Rose Lampert Graff Foundation, Los Angeles, Calif.

[1] Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., Proc. Soc. Exp. Biol. and Med., 1948, 68, 1.

[2] Nicholls, E. E., and Stainsby, W. J., *J. Clin. Invest.*, 1931, 10, 323.

[3] Dawson, M. H., Olmstead, M., and Boots, R. H., *J. Immunol.*, 1932, 23, 187.

[4] Hench, P. S., Oxford Univ. Press, 1938, p. 35.

[5] Wallis, A. D., *Am. J. Med. Sc.*, 1946, 212, 713, 716, 718.

[6] Sales Gomes, L., and Brito e Silva, M., *Revista do Inst. Adolfo Lutz*, 1942, 2, 212; *ibid.*, 1943, 3, 25.

[†] This observation, incidentally, has not been confirmed in this laboratory.

TABLE I.

Results of Differential Sheep Cell Agglutination Tests on Serum Specimens from Patients with Rheumatoid Arthritis and a Variety of Other Conditions.

Clinical diagnosis*	No. of cases	Distribution of cases according to differential agglutination titer									
		0	2	4	8	16	32	64	128	256	512
Rheumatoid arthritis	35	1	8	6	4	6	5	4			1
Rheumatoid spondylitis (Marie-Strümpell)	7		4	1	1†	1†					
Reiter's disease	1		1								
Arthralgia	1		1								
Osteo-arthritis	3	1	1		1						
Gonorrheal arthritis (?)	1		1								
Scleroderma	3		1		2						
Lupus erythematosus disseminatus	1		1								
Acute rheumatic fever	18	9	8	1							
Infectious mononucleosis	2	2									
Lymphogranuloma venereum	14		11	3							
Syphilis	17	5	10	2							
Infectious diseases without evidence of arthritis‡	18	4	9	5							
Other diseases without evidence of arthritis§	9	2	6	1							
Presumably normal persons	18	3	13	10	2						

* In all instances the clinical diagnosis was substantiated by appropriate laboratory tests when availble.

† This patient also had peripheral joint involvement.

‡ These include one case each of brucellosis, infectious hepatitis, equine encephalomyelitis (Eastern type), primary atypical pneumonia, typhus fever, pneumococcal pneumonia, and poliomyelitis, 2 cases each of psittacosis and toxoplamosis, 3 cases of lymphocytic choriomeningitis, and 4 cases of Q fever.

§ These include one case each of acute glomerulonephritis and Hodgkin's disease, 2 cases each of myelogenous leukemia, carcinoma and multiple myeloma.

TABLE II.

Results of Differential Sheep Cell Agglutination Tests on Serum Specimens from Patients with Rheumatoid Arthritis Classified According to Severity and Duration of Illness.*

Severity of disease	Duration of illness (yrs)	No. of cases	Distribution of cases according to differential agglutination titer									
			0	2	4	8	16	32	64	128	256	512
Mild	<1	2			2							
	1–5	3		2	1							
	>5	0										
Moderate	<1	3	1	1	1							
	1–5	10		2		4	2	2				
	>5	8		1	2		4		1			
Marked	<1	0										
	1–5	2						1	1			
	>5	7		2†				2	2			1

* Not including 7 cases with evidence of rheumatoid spondylitis (Marie-Strümpell).

† One of these is an inactive case of long duration, the other is a case of juvenile rheumatoid arthritis (Still's disease).

ing these patients: intensity of constitutional symptoms, speed of progression of illness, degree of disability, degree and extent of joint swelling, amount of bone atrophy and destruction, and activity evidenced by results of erythrocyte sedimentation tests.

It is evident from the data presented in Table II that none of the patients classified as having mild rheumatic arthritis and none of those with a disease of moderate severity of less than one year duration showed differential agglutination titers greater than 4. Only 2 patients with a disease of marked severity had a differential agglutination titer less than 32. One of these patients with rheumatoid arthritis for a period of 30 years was "inactive" when the blood specimen was obtained. The other was a case of juvenile rheumatoid arthritis (Still's disease). Thirteen of the 18 cases with an illness of moderate severity of more than one year duration had differential agglutination titers of 8 or greater. The distribution of cases according to the differential agglutination titer would seem to reflect the clinical severity of the disease.

Many of the patients with high differential agglutination titers also had high sedimentation rates. There was no correlation, however, between the height of the sedimentation rate and the result of the differential sheep cell agglutination test, since some patients whose serum showed a differential agglutination titer of less than 16 had high sedimentation rates. This, likewise, is in accord with observations reported previously.[1]

A second blood specimen was obtained from 5 of the patients with rheumatoid arthritis after an interval of about one month. One original differential agglutination titer of 512, one of 64, and two of 4 were found to be the same, while one titer of 16 was 8 on the second examination. Further follow-up studies are contemplated with the view to elucidating the mechanism of this phenomenon. Data thus far available indicate that the differential sheep cell agglutination test is of limited value as an aid in the diagnosis of active rheumatoid arthritis of mild severity.

Summary. 1. The differential sheep cell agglutination test of Rose and his associates was performed with serum specimens from 42 individuals with rheumatoid arthritis. Serum from 17 of these patients showed differential agglutination titers of 16 or greater. In 24 active cases of mild and moderate severity and in one inactive case of long duration a differential agglutination titer of less than 16 was obtained.

2. Sera from 88 individuals with a variety of other conditions and from 18 presumably normal persons tested in the same manner showed differential agglutination titers less than 16.

3. Although differential agglutination titers less than 16 were observed in more than half the cases of active rheumatoid arthritis, the

test appears to reflect the clinical severity of the disease.

4. These data partially confirm the observations of Rose and his associates but indicate that the differential sheep cell agglutination test is of limited value as an aid in the diagnosis of active rheumatoid arthritis of mild severity.

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IV. Ineffectiveness of Certain Essential Nutrients in Prevention of Tooth Decay in Cotton Rat Molars.

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The molar teeth of cotton rats (*Sigmondon hispidus hispidus*) have been found to be susceptible to the initiation and development of carious lesions when the rats were maintained on purified rations for 10 weeks or more after weaning.[1,2] The basal rations used in these studies were believed to be adequate in known essential nutrients in so far as the growth and maintenance of rodents were concerned. When the fat, or the protein content, or both, were increased isocalorically at the expense of sucrose, weanling cotton rats fed the resulting rations developed a much lower average number and average extent of carious lesions than the littermates fed the basal ration for the same period.[3,4] Before extensive use of this ration and its various modifications in the investigation of the effect of diet during or after tooth development on the susceptibility to tooth decay, it seemed mandatory to determine whether superabundant amounts of known essential nutrients, or the same nutrients from different sources, would alter the initiation and development of carious lesions in cotton rats when the molar teeth were fully formed and were known to be highly susceptible to dental caries at the beginning of the experimental period.

Experimental. The purified ration (100) used in most of these studies has been described in detail.[5] In a few of these studies, a slight modification (130) of the basal ration was used which was made by the isocaloric replacement of 18 of each 67 g of sucrose with 8 g of lard. Both diets were nutritionally adequate for the production of normal growth and development of the cotton rat.

The cotton rats used in these investigations were raised in our stock colony and weaned at about 21 days of age. At this time, the first and second molars of the cotton rat are fully erupted and the crowns of the third molars are largely formed but have not erupted into the oral cavity. Experimental procedure was identical with that described for previous experiments.[5]

In the first experiment, a series of 7 groups of cotton rats was fed various combinations of water-soluble and fat-soluble vitamins in superabundant amounts as described in Table I. Parallel to each experimental group, a comparable group of control littermates was maintained on ration 100, except in the seventh group where ration 100 without liver concentrates was used.

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[1] Shaw, J. H., Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Phillips, P. H., *J. Nutrition*, 1944, 28, 333.

[2] Shaw, J. H., Schweigert, B. S., Elvehjem, C. A., and Phillips, P. H., *J. Dental Research*, 1944, 23, 47.

[3] Schweigert, B. S., Shaw, J. H., Zepplin, M., and Elvehjem, C. A., *J. Nutrition*, 1946, 31, 439.

[4] Schweigert, B. S., Potts, E., Shaw, J. H., Zepplin, M., and Phillips, P. H., *J. Nutrition*, 1946, 32, 405.

[5] Shaw, J. H., *J. Dental Research*, 1947, 26, 47.

TABLE I. Effect of Additions of Various Water-Soluble, Fat-Soluble Vitamins on Average Number, Average Extent Carious Lesions in Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	7	29.6	2.1	.3	79+	10+	.4
100 + B-complex‡	7	28.9	1.8		86+	12+	
100	5	26.8	1.7	1.2	83+	7+	1.8
100 + 0.4% ascorbic acid	5	23.6	2.1		65+	7+	
100	5	24.8	4.2	.6	67+	18+	.2
100 + B-complex‡ + 0.4% ascorbic acid	7	27.6	2.7		71+	16+	
100	10	26.1	2.4	.3	73+	11+	.4
100 + ADEK§	11	27.1	2.6		78+	10+	
100	4	24.5	2.6	.9	74+	12+	.6
100 + 0.1% menadione	5	27.2	1.4		84+	9+	
100	9	33.0	1.0	1.2	110+	6+	.6
100 + 0.1% natural vitamin K_1	10	31.3	1.0		104+	8+	
100	12	26.7	1.1	.3	75+	6+	.4
100 + liver concentrates + 200 µg folic acid	11	27.2	1.0		79+	7+	

* Standard error of mean. † Critical ratio.

‡ To each kg of ration 100 were added an additional 10.5 mg of thiamine hydrochloride, 10.5 mg of riboflavin, 10.5 mg of pyridoxine hydrochloride, 75 mg of nicotinic acid, 60 mg of calcium pantothenate, 3.0 g of choline chloride, 3.0 g of inositol, and .9 g of para-aminobenzoic acid, *i.e.*, enough of each to make the final concentration 4 times the amounts normally supplied. In addition, 200 µg of biotin were added per kilo.

§ To each kg of ration 100 were added an additional 33 mg of beta-carotene, 9000 I.U. of irradiated ergosterol, 18 mg of 2-methyl, 1,4-naphthoquinone, and 150 mg of alpha-tocopherol, *i.e.*, enough of each to make the final concentration 4 times the amounts ordinarily supplied.

TABLE II. Effect of the Replacement of Purified Casein by Crude Casein, Blood Fibrin, and Lactalbumin and of Additions of *dl*-Tryptophane on the Average Number and Average Extent of Carious Lesions in the Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	4	37.0	.9	.3	120+	6+	1.2
400 (crude casein)	4	35.8	3.0		104+	10+	
130	3	24.3	3.8	.7	57+	17+	.3
430 (crude casein)	3	21.0	2.9		51+	13+	
100	8	29.5	1.3	2.0	83+	7+	2.0
200 (blood fibrin)	8	32.5	.7		102+	6+	
130	8	18.0	2.5	.5	41+	10+	.3
230 (blood fibrin)	9	17.3	2.3		36+	8+	
100	10	32.2	1.2	.9	104+	7+	1.1
300 (lactalbumin)	10	30.1	1.9		90+	10+	
130	6	19.5	1.9	1.5	41+	9+	1.1
330 (lactalbumin)	6	24.5	2.7		57+	11+	
100	9	27.8	2.0	1.1	77+	9+	.6
100 + 0.5% *dl*-tryptophane	9	25.2	1.4		70+	8+	

* Standard error of mean. † Critical ratio.

TABLE III.

Effect of Mineral Alterations on the Average Number and Average Extent of Carious Lesions in the Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	7	27.6	1.8	.9	85+	10+	.9
100 + 2% salt mixt.	7	25.7	1.1		75+	6+	
100	9	29.4	1.4	.6	96+	6+	1.1
1100 (sol. salt mixt.)‡	9	28.3	1.1		87+	6+	
100	6	28.7	1.0	1.2	86+	10+	1.2
1100 + 2% sol.‡ salt mixt.	5	24.4	3.1		69+	10+	
100	7	24.3	2.8	1.5	69+	11+	1.0
100 + 0.5% sodium oxalate	9	19.3	1.9		55+	8+	
100	5	23.8	1.0	1.9	64+	11+	1.0
100 + 1.0% sodium oxalate	6	18.2	2.9		48+	12+	

* Standard error of mean

† Critical ratio.

‡ Calcium lactate 400 g, anhydrous calcium chloride 710 g, monosodium phosphate 1050 g, anhydrous sodium carbonate 207 g, anhydrous potassium carbonate 480 g, anhydrous magnesium sulfate 100 g, ferric citrate 55 g, potassium iodide 1.0 g, manganese sulfate 9.0 g, anhydrous zinc chloride 4.0 g, anhydrous cupric sulfate 2.4 g, and cobaltous sulfate 0.2 g.

TABLE IV.

Average Number and Extent of Carious Lesions Observed in Cotton Rats Maintained for 14 Weeks on Ration 100, Keyes' Hamster Ration, and on Ration 100 Plus Yeast.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	4	33.5	1.5	5.3	100+	7+	7.0
Keyes'	5	10.8	2.6		30+	8+	
100-liver	7	33.7	1.4	3.0	110+	9+	2.1
100-liver + 8% brewer's yeast	8	28.0	1.5		80+	8+	

*Standard error of mean.

† Critical ratio.

TABLE I. Effect of Additions of Various Water-Soluble, Fat-Soluble Vitamins on Average Number, Average Extent Carious Lesions in Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	7	29.6	2.1	.3	79+	10+	.4
100 + B-complex‡	7	28.9	1.8		86+	12+	
100	5	26.8	1.7	1.2	83+	7+	1.8
100 + 0.4% ascorbic acid	5	23.6	2.1		65+	7+	
100	5	24.8	4.2	.6	67+	18+	.2
100 + B-complex‡ + 0.4% ascorbic acid	7	27.6	2.7		71+	16+	
100	10	26.1	2.4	.3	73+	11+	.4
100 + ADEK§	11	27.1	2.6		78+	10+	
100	4	24.5	2.6	.9	74+	12+	.6
100 + 0.1% menadione	5	27.2	1.4		84+	9+	
100	9	33.0	1.0	1.2	110+	6+	.6
100 + 0.1% natural vitamin K_1	10	31.3	1.0		104+	8+	
100	12	26.7	1.1	.3	75+	6+	.4
100 + liver concentrates + 200 μg folic acid	11	27.2	1.0		79+	7+	

* Standard error of mean. † Critical ratio.

‡ To each kg of ration 100 were added an additional 10.5 mg of thiamine hydrochloride, 10.5 mg of riboflavin, 10.5 mg of pyridoxine hydrochloride, 75 mg of nicotinic acid, 60 mg of calcium pantothenate, 3.0 g of choline chloride, 3.0 g of inositol, and .9 g of para-aminobenzoic acid, *i.e.*, enough of each to make the final concentration 4 times the amounts normally supplied. In addition, 200 μg of biotin were added per kilo.

§ To each kg of ration 100 were added an additional 33 mg of beta-carotene, 9000 I.U. of irradiated ergosterol, 18 mg of 2-methyl, 1,4-naphthoquinone, and 150 mg of alpha-tocopherol, *i.e.*, enough of each to make the final concentration 4 times the amounts ordinarily supplied.

TABLE II. Effect of the Replacement of Purified Casein by Crude Casein, Blood Fibrin, and Lactalbumin and of Additions of *dl*-Tryptophane on the Average Number and Average Extent of Carious Lesions in the Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	4	37.0	.9	.3	120+	6+	1.2
400 (crude casein)	4	35.8	3.0		104+	10+	
130	3	24.3	3.8	.7	57+	17+	.3
430 (crude casein)	3	21.0	2.9		51+	13+	
100	8	29.5	1.3	2.0	83+	7+	2.0
200 (blood fibrin)	8	32.5	.7		102+	6+	
130	8	18.0	2.5	.5	41+	10+	.3
230 (blood fibrin)	9	17.3	2.3		36+	8+	
100	10	32.2	1.2	.9	104+	7+	1.1
300 (lactalbumin)	10	30.1	1.9		90+	10+	
130	6	19.5	1.9	1.5	41+	9+	1.1
330 (lactalbumin)	6	24.5	2.7		57+	11+	
100	9	27.8	2.0	1.1	77+	9+	.6
100 + 0.5% *dl*-tryptophane	9	25.2	1.4		70+	8+	

* Standard error of mean. † Critical ratio.

TABLE III.

Effect of Mineral Alterations on the Average Number and Average Extent of Carious Lesions in the Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	7	27.6	1.8	.9	85+	10+	.9
100 + 2% salt mixt.	7	25.7	1.1		75+	6+	
100	9	29.4	1.4	.6	96+	6+	1.1
1100 (sol. salt mixt.)‡	9	28.3	1.1		87+	6+	
100	6	28.7	1.9	1.2	86+	10+	1.2
1100 + 2% sol.‡ salt mixt.	5	24.4	3.1		69+	10+	
100	7	24.3	2.8	1.5	69+	11+	1.0
100 + 0.5% sodium oxalate	9	19.3	1.9		55+	8+	
100	5	23.8	1.9	1.9	64+	11+	1.0
100 + 1.0% sodium oxalate	6	18.2	2.2		48+	12+	

* Standard error of mean

† Critical ratio.

‡ Calcium lactate 490 g, anhydrous calcium chloride 710 g, monosodium phosphate 1050 g, anhydrous sodium carbonate 207 g, anhydrous potassium carbonate 480 g, anhydrous magnesium sulfate 100 g, ferric citrate 55 g, potassium iodide 1.6 g, manganese sulfate 9.0 g, anhydrous zinc chloride 4.0 g, anhydrous cupric sulfate 2.4 g, and cobaltous sulfate 0.2 g.

TABLE IV.

Average Number and Extent of Carious Lesions Observed in Cotton Rats Maintained for 14 Weeks on Ration 100, Keyes' Hamster Ration, and on Ration 100 Plus Yeast.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
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Keyes'	5	16.8	2.6		36+	8+	
100-liver	7	33.7	1.4	3.0	110+	6+	2.1
100-liver + 8% brewer's yeast	8	28.0	1.5		89+	8+	

*Standard error of mean.

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100	5	26.8	1.7	1.2	83+	7+	1.8
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100	9	33.0	1.0	1.2	110+	6+	.6
100 + 0.1% natural vitamin K_1	10	31.3	1.0		104+	8+	
100	12	26.7	1.1	.3	75+	6+	.4
100 + liver concentrates + 200 µg folic acid	11	27.2	1.0		79+	7+	

* Standard error of mean. † Critical ratio.

‡ To each kg of ration 100 were added an additional 10.5 mg of thiamine hydrochloride, 10.5 mg of riboflavin, 10.5 mg of pyridoxine hydrochloride, 75 mg of nicotinic acid, 60 mg of calcium pantothenate, 3.0 g of choline chloride, 3.0 g of inositol, and .9 g of para-aminobenzoic acid, *i.e.*, enough of each to make the final concentration 4 times the amounts normally supplied. In addition, 200 µg of biotin were added per kilo.

§ To each kg of ration 100 were added an additional 33 mg of beta-carotene, 9000 I.U. of irradiated ergosterol, 18 mg of 2-methyl, 1,4-naphthoquinone, and 150 mg of alpha-tocopherol, *i.e.*, enough of each to make the final concentration 4 times the amounts ordinarily supplied.

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130	3	24.3	3.8	.7	57+	17+	.3
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100	8	29.5	1.3	2.0	83+	7+	2.0
200 (blood fibrin)	8	32.5	.7		102+	6+	
130	8	18.0	2.5	.5	41+	10+	.3
230 (blood fibrin)	9	17.3	2.3		36+	8+	
100	10	32.2	1.2	.9	104+	7+	1.1
300 (lactalbumin)	10	30.1	1.9		90+	10+	
130	6	19.5	1.9	1.5	41+	9+	1.1
330 (lactalbumin)	6	24.5	2.7		57+	11+	
100	9	27.8	2.0	1.1	77+	9+	.6
100 + 0.5% *dl*-tryptophane	9	25.2	1.4		70+	8+	

* Standard error of mean. † Critical ratio.

significance in the average number and average extent of the carious lesions.

Summary. A variety of tests were made to determine if superabundant amounts of essential nutrients or optimal amounts of these nutrients from different sources would alter the initiation and development of carious lesions in caries-susceptible cotton rats after tooth development was almost complete. Negative results were obtained in all cases indicating that initiation and development of carious lesions in teeth formed before the beginning of the experimental period were not influenced by additional amounts of the known nutrients tested.

The ration described by Keyes for production of carious lesions in hamsters resulted in very poor growth and a low rate of initiation and development of carious lesions in the cotton rat. A supplement of 8% dried brewer's yeast to the purified ration produced good growth and permitted a slight reduction in the dental caries experience of cotton rats.

This project was supported in part by a grant from the Sugar Research Foundation, Inc., New York. We are indebted to Merck & Co., Inc., Rahway, N.J., for gifts of the water-soluble vitamins used in these experiments.

16966

Tuberculostatic Effect of Furacin *in vitro* and *in vivo*.*

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Furacin is a nitrofuran compound (5-nitro-2-furaldehyde semicarbazone) which has definite chemotherapeutic effects against certain infections *in vivo*, as well as bacteriostatic and bactericidal properties against gram-positive and gram-negative organisms in the test tube.[1-7]

This report presents the results of experiments designed to show the effect of Furacin on the growth of tubercle bacilli in the test tube, and on a tuberculous infection in guinea pigs.

To determine the influence of Furacin on the growth of the human type H37 Rv microörganisms, 4 different fluid media were employed: Proskauer and Beck's synthetic medium, Proskauer and Beck's medium with 10% human serum, Kirschner's synthetic medium, and the Tween-albumin medium of Dubos. They were dispensed in 5 cc amounts in 18x150 mm test tubes. The inoculum into each tube was 0.1 cc of a 7-day culture of H37 Rv microörganisms (about 0.03 mg dry weight) in Tween-albumin liquid medium. All determinations were made in triplicate.

Table I reveals that Furacin in a concentration of 1:40,000 produced a slight inhibition of growth of the organisms, whereas in a concentration of 1:20,000 it prevented any appreciable growth for 28 days.

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* The Furacin was supplied through the courtesy of Dr. L. Eugene Daily of the Eaton Laboratories, Norwich, N. Y. (Lot No. 1050).

[1] Dodd, M. C., and Stillman, W. B., *J. Pharm. and Exp. Therap.*, 1944, **82**, 11.

[2] Snyder, M. L., Kiehn, C. L., and Christopherson, J. W., *Mil. Surgeon*, 1945, **97**, 380.

[3] Cramer, D. L., and Dodd, M. C., *J. Bact.*, 1946, **51**, 293.

[4] Neter, E., and Lamberti, T. G., *Am. J. Surg.*, 1946, **72**, 246.

[5] Dodd, M. C., *J. Pharm. and Exp. Therap.*, 1946, **86**, 311.

[6] Shipley, E. R., and Dodd, M. C., *Surg., Gynec., and Obst.*, 1947, **84**, 366.

[7] Green, M. N., and Mudd, Stuart, PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 57.

In the second experiment, crude casein, blood fibrin, and lactalbumin were used as individual protein sources by the complete replacement of the "de-vitaminized" casein in ration 100, or in ration 130. In addition, the effect of a supplement of 0.5% *dl*-tryptophane to ration 100 was tested.

In the third experiment, a series of mineral alterations was made, as detailed in Table III.

In the fourth experiment, one group of experimental cotton rats was fed the ration* used by Keyes[6] for the production of carious lesions in the hamster. A second group of cotton rats was fed ration 100 with the liver concentrates replaced by 8% dried brewer's yeast.

Results. The results of the first experiment in which additional amounts of the water-soluble and fat-soluble vitamins were added to ration 100 throughout the 14-week experimental period are summarized in Table I. In all cases, the additional amounts of these nutrients tested, after development of the teeth was largely completed, did not significantly alter the average number nor the average extent of carious lesions. The rate of increase in body weight was not altered by any of these supplements.

The results of the second experiment, in which the "de-vitaminized" casein in rations 100 or 130 was completely replaced by crude casein, blood fibrin, or lactalbumin, and where ration 100 was supplemented by 0.5% *dl*-tryptophane, are presented in Table II. The use of crude casein, blood fibrin, or lactalbumin did not alter the susceptibility of these cotton rats to develop carious lesions under the circumstances in this experimental regimen. Likewise, a supplement of 0.5% *dl*-tryptophane did not alter the initiation and development of carious lesions when fed after tooth development was complete. Increases in body weight were not altered by these supplements, except in the case of those cotton rats fed blood fibrin. These animals gained slightly more rapidly during the early weeks of the experiment and attained slightly higher average final body weights.

The results of the mineral alterations in the third experiment are presented in Table III. A supplement of an additional 2% of the regular salt mixture, and the replacement of the regular salt mixture by a more soluble salt mixture at 3 and 5% did not alter the susceptibility to tooth decay when fed after tooth development was largely finished. The supplements of both 0.5 and 1.0% sodium oxalate to ration 100 seemed to reduce the average number of carious lesions and the average extent slightly, but not sufficiently to be judged statistically significant by the test used. Both groups of cotton rats fed sodium oxalate had a severe and prolonged diarrhea and did not gain weight as rapidly as their controls nor attain as high final body weights. These factors may have been responsible to some degree for any tendency to reduce the development of carious lesions.

When cotton rats were fed a ration described by Keyes[6] for production of a high incidence of carious lesions in the hamster, the average number and average extent of carious lesions were significantly lower than for littermates fed ration 100 during the same period (Table IV). Those lesions which did develop in cotton rats fed Keyes' ration were characteristic in location and in type with those observed in cotton rats after only 6 to 10 weeks on experiment. Unlike the hamster, no lesions were produced on the smooth surfaces of the molar teeth in the cotton rat. One possible explanation for the reduced dental caries attack rate in cotton rats fed Keyes' ration may be based upon the low food intake and low rate of growth observed. Due to the apparent dislike of this ration by cotton rats, more ration was scattered and wasted than was eaten. A similar effect of enforced food restriction on the initiation and development of carious lesions has been described previously.[7]

The replacement of the liver concentrates in ration 100 by 8% of brewer's yeast resulted in a small reduction, bordering on statistical

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* Whole wheat flour 20%, cornstarch 25%, confectionery sugar 20%, whole powdered milk 30%, and ground alfalfa 5%.

[6] Keyes, P. H., *J. Dental Research*, 1946, **25**, 341.

[7] Shaw, J. H., *J. Am. Diet. Assn.*, 1948, **24**, 181.

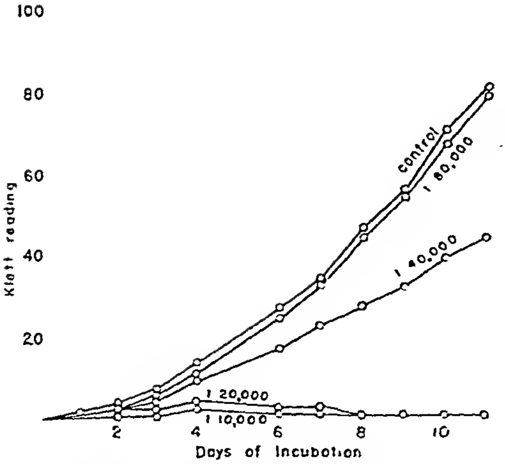

FIG. 1.
Effect of Furacin on growth of H37 "Rv" in Tween albumin medium.

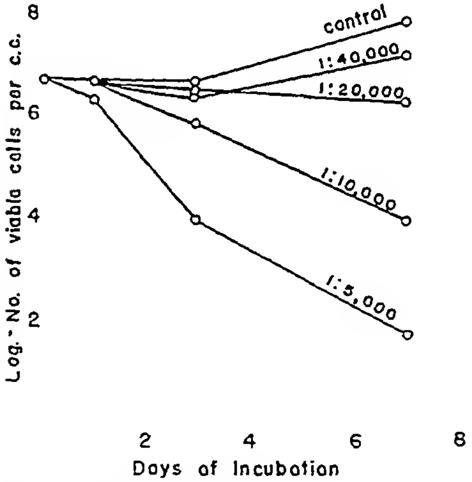

FIG. 2.
Bactericidal action of Furacin on H37 "Rv" in Tween albumin medium.

16 such transfers, growth of the culture was still inhibited by a 1:20,000 dilution of the drug.

The possible inhibitory effect of Furacin on the development of streptomycin resistance was tested *in vitro*. A sufficient amount of Furacin to make a final concentration of 1:50,000 was added to Tween-albumin liquid medium containing graded concentrations of streptomycin. Serial transfers of the H37 Rv culture were made to progressively higher concentrations of streptomycin in the usual manner, and the rate at which resistance developed was compared with a similarly handled culture in the absence of Furacin. It was found that the presence of Furacin in the medium delayed, but did not prevent, the development of streptomycin resistance by the tubercle bacilli.

In Vivo Tests in Guinea Pigs. Before initiating drug therapy, the toxicity of Furacin for guinea pigs was investigated. The drug was suspended in 10% gum acacia in a concentration of 150 mg per cc, and fed to normal pigs (average weight, 500 g) by means of a tuberculin syringe fitted with a blunt needle. It was found that 150 mg once a day, or 75 mg twice a day, killed all the animals used in the test within a period of 5 days. Guinea pigs fed 75 mg per day, either in 1 dose or 2, appeared very ill by the 10th day, and it was obvious that all the animals would have died shortly. The dosage was reduced to 37.5 mg once a day for 10 days. This treatment period was followed by a rest period of 10 days, after which time the drug was resumed. Three animals survived 28, 32, and 44 days, respectively, before succumbing to the toxic effects of the drug.

The major experiment was then begun. Thirty-five guinea pigs were infected with the human type H37 Rv culture. Each animal received 0.05 mg (dry weight) subcutaneously in the inguinal region. They were of mixed sexes and weighed between 600 and 900 g. Ten were kept as untreated controls, and 25 were started on Furacin treatment 6 days after infection.

The drug was suspended in 10% gum acacia in concentration of 75 mg per cc. Each treated animal was fed 18.75 mg at 12-hour intervals. After 11 days of treatment, several animals appeared listless and refused to eat. By the 13th day, 6 guinea pigs had died, and treatment was suspended for 17 days. Despite this rest period, another 5 animals died during the next 6 days, evidently of Furacin toxicity.

When Furacin was resumed 17 days later, the pigs had recovered sufficiently to appear healthy and to have good appetites again. The drug was then given in dosage of 18.75 mg once a day until the experiment was terminated by sacrificing all survivors 70

TABLE I.

Effect of Furacin on the Growth of H37Rv Tubercle Bacilli in Four Different Types of Fluid Medium.

A. Proskauer and Beck's Synthetic Medium.
Amt of growth in days

Furacin Conc.	8	12	16	21	28
Control	+	+	+	2+	2+
1:80×1000	+	+	+	2+	2+
1:40 ”	0	±	+	+	2+
1:20 ”	0	0	0	0	0
1:10 ”	0	0	0	0	0
1: 5 ”	0	0	0	0	0

B. Proskauer and Beck's with 10% Human Serum.
Amt of growth in days

Furacin Conc.	4	8	12	16	22
Control	+	2+	3+	4+	4+
1:80×1000	±	2+	3+	3+	3+
1:40 ”	±	+	2+	2+	3+
1:20 ”	0	±	±	±	±
1:10 ”	0	±	±	±	±
1: 5 ”	0	0	0	0	0

C. Kirschner's Synthetic Medium.
Amt of growth in days

Furacin Conc.	8	12	16	21	28
Control	3+	3+	4+	4+	4+
1:80×1000	2+	2+	3+	4+	4+
1:40 ”	+	+	2+	2+	2+
1:20 ”	±	±	±	±	±
1:10 ”	0	±	±	±	±
1: 5 ”	0	0	0	0	0

D. Tween-albumin Liquid Medium.
Amt of growth in days

Furacin Conc.	4	8	12	16	22
Control	2+	4+	4+	4+	4+
1:80×1000	+	3+	4+	4+	4+
1:40 ”	±	2+	3+	4+	4+
1:20 ”	0	0	0	±	±
1:10 ”	0	0	0	0	0
1: 5 ”	0	0	0	0	0

Using an inoculum of 0.2 cc (twice the original amount), it was found that a 1:20,000 concentration of Furacin still inhibited growth of the organisms. The H37 Rv culture which had been made resistant to streptomycin exhibited the same sensitivity to Furacin as the original streptomycin-sensitive strain.

Fig. 1 illustrates the effect of different concentrations of Furacin on the turbidity produced in Tween-albumin medium by the H37 Rv culture, as determined in the Klett-Summerson colorimeter. The technic was the same as that described previously for streptomycin.[8] Here again, it will be observed that Furacin in a 1:40,000 dilution produced slight inhibition of growth of the organisms, whereas the drug in a 1:20,000 dilution prevented the development of turbidity for 11 days.

Furacin exerts a bactericidal action on tubercle bacilli, as well as a growth-inhibiting action. This was illustrated by inoculating 0.5 cc of a culture of H37 Rv into 25 cc of Tween-albumin medium in a small flask, which was then incubated at 37°C. Similar flasks were set up, containing Furacin in dilutions of 1:40,000, 1:20,000, 1:10,000 and 1:5,000. At various intervals, samples were removed, appropriately diluted with Tween-albumin medium, and 0.1 cc amounts spread evenly over the surface of a solid egg-yolk, potato medium contained in flat glass tubes. Each dilution was planted in triplicate. Colony counts were made after incubation of the slants (in a horizontal position) for 30 days.

Fig. 2 illustrates the results of the experiment. Furacin in a concentration of 1:20,000 produced only a small decrease in the number of viable cells after 7 days, but in a concentration of 1:5,000 the drug had rendered non-viable the vast majority of tubercle bacilli by the 7th day.

In contrast to the bactericidal effect of Furacin on multiplying bacilli, there was no killing action whatsoever when the flasks were kept in the refrigerator at 5°C.

Tubercle bacilli of the H37 Rv strain could not be made resistant to the action of Furacin in the test tube. This culture was inoculated into a series of tubes of Tween-albumin medium containing the following concentrations of the drug: 1:80,000, 1:40,000, 1:20,000, 1:10,000, and 1:5000. After 10 to 12 days' incubation the medium in the tube containing the highest concentration of Furacin which allowed growth was used to inoculate a similar series of Furacin dilutions. At the end of this failure *in vivo*. Furacin is relatively insoluble and must be administered as a suspension of large particles which tend to settle out easily. It is not known how much is absorbed or what happens to the compound in the body. No Furacin can be demonstrated in the blood of animals to whom the drug has been given, and only a very small amount in the urine.[9] It is not known how much of the drug is converted to an inactive compound in the body of the guinea pig.

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[8] Wolinsky, E., and Steenken, W., Jr., *Am. Rev. Tuberc.*, 1947, **55**, 281.

The best chemotherapeutic results in mice infected with Staphylococci, Streptococci, and Salmonella organisms were obtained by feeding 150 to 200 mg per kg daily for 3 days.[5] It was not possible to administer this amount of Furacin to guinea pigs without fatal results, and it was necessary to decrease the dose to about 35 mg per kg daily, which seemed to be the maximum tolerated dose for prolonged therapy. This relatively small amount of Furacin was probably not sufficient to produce a bacteriostatic or bactericidal level in the body of the guinea pig.

It was observed that the toxic effects of Furacin continued to be manifest for many days after the administration of the drug was stopped. On the regimen of 18.75 mg per day, the guinea pigs appeared healthy for 10 days, but then almost all began to show loss of appetite, listlessness, and loss of weight. Deaths started to occur on the 11th day. Even after the drug was withdrawn, some animals continued to decline and die for the next 5 or 6 days, before improvement in the appearance of the group was noted.

Conclusions. 1. Furacin exerts a bacteriostatic and bactericidal action on virulent human tubercle bacilli *in vitro*. In a concentration of 1:20,000 (5 mg %) it prevented growth for 22 days in 4 different types of fluid media. A concentration of 1:5000 (20 mg%) was required for bactericidal effect.

2. Resistance to Furacin was not evident after many transfers of the H37 Rv culture in drug-containing medium.

3. The combination of Furacin and streptomycin in Tween-albumin liquid medium delayed, but did not prevent, the development of streptomycin-resistance by the H37 Rv tubercle bacillus.

4. Furacin, in the maximum tolerated dose of about 35 mg per kg daily for 64 days, did not appreciably affect the course of tuberculosis produced in guinea pigs by the human type H37 Rv culture.

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[9] Unpublished results, Eaton Research Laboratories.

16967

Effects of Adding Carbon Dioxide to Inspired Oxygen on Tolerance to High Altitudes.*

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When CO_2 is added to the inspired air at altitudes around 20,000 feet or to low oxygen mixtures, there is an improvement in the physiological condition which is due to an increase in the alveolar pO_2.[1-4] There is no general agreement, however, on the question

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[*] Work done under contract recommended by Committee on Medical Research between the Office of Scientific Research and Development and the University of Rochester, and under contract with Air Materiel Command, Wright Field.

[1] Fenn, W. O., Rahn, H., and Otis, A. B., *Am. J. Physiol.*, 1946, **146**, 637.

[2] Rahn, H., and Otis, A. B., *Am. J. Physiol.*, 1947, **150**, 202.

[3] Gray, J. S., A.A.F. School of Aviation Medicine, Randolph Field, Project Report 310, August, 1944.

[4] Gibbs, F. A., Gibbs, E. L., Lennox, W. G., and Nims, L. F., *J. Av. Med.*, 1943, **14**, 250.

TABLE II.
Results in Guinea Pig Tuberculosis Treated with Furacin.

G. pig No.	Fate	Days of infection	Days of* treatment	Amt of tuberculosis grossly at autopsy
Controls				
1	Died	19	0	6+
2	"	63	0	9.5+
3	"	66	0	7.5+
4	"	67	0	14+
5	Killed	71	0	10+
6	"	"	0	9+
7	"	"	0	11+
8	"	"	0	8+
9	"	"	0	9+
10	"	"	0	7.5+
				Avg‡ 9.1+
Treated				
1	Died	13	7	0
2	"	17	11	1+
3	"	18	12	4+
4	"	"	"	5.5+
5	"	19	13	3+
6	"	"	"	3+
7	"	21	15	4.5+
8	"	22	16	3+
9	"	23	17	3+
10	"	25	19	4+
11	"	"	"	4.5+
12	"	53	47	12+
13	"	56	50	6+
14	"	61	55	7+
15	"	67	61	7+
16	Killed	70	64	10+
17	"	"	"	6.5+
18	"	"	"	7+
19	"	"	"	12+
20	"	"	"	10.5+
21	"	"	"	11+
22	**	"	"	10+
23	"	"	"	10+
24	"	"	"	9.5+
25	"	"	"	9.5+
				Avg‡ 9.6+

* Including rest period of 17 days without Furacin after the first 13 days of treatment.
‡ Average of those animals killed at the termination of the experiment.

days after infection, or 64 days after Furacin treatment was begun.

The results of this experiment are shown in Table II. The amount of tuberculous disease in the spleen, liver, lungs, and lymph nodes of each pig was graded from zero to 4+, according to the gross appearance. Thus, the maximum disease for an animal was 16+. The average degree of tuberculosis of the 6 controls which survived the full period of 70 days was 9.1+, whereas the corresponding figure for the 10 Furacin-treated animals which were killed at the termination of the experiment was 9.6+ It was thus *demonstrated* that Furacin, in the comparatively low dosage necessary because of the evident toxicity for guinea pigs, had no tuberculostatic effect on this infection produced by H37 Rv organisms.

Discussion. The experiments herein described reveal that Furacin is a compound which exerts definite bacteriostatic and bactericidal action on virulent human tubercle bacilli in the test tube; upon the progression of a tuberculous infection in guinea pigs, however, the drug appears to have no inhibitory effect.

There are some possible explanations for

TABLE I.

Series	Gas mixture	Mean ceiling altitude, 10^3 ft	Standard deviation, 10^3 ft	S.D. of mean, 10^3 ft	No. of mouse runs	No. of mice
A	100% O_2	50.3	2.1	.33	41	6
	21.7% CO_2	50.9	2.4	.39	38	6
	20% N_2	49.5	2.1	.36	35	6
B	100% O_2	48.6	1.3	.19	50	15
	10.5% CO_2	47.4	1.6	.24	45	15
	21.7% CO_2	46.8	1.6	.22	53	15
C	100% O_2	48.6	1.3	.24	28	15
	10.5% CO_2	48.4	1.1	.19	29	15
D	100% O_2	53.4	2.5	.43	34	11
	10.5% CO_2	51.3	2.5	.42	36	11

TABLE II.
Summary of Measurements on Human Subjects Breathing 100% O_2 and 90% O_2-10% CO_2 at 40,000 Feet.

	Min. vol., liters/min. B.T.P.S.		Oximeter, % saturation		Alveolar pCO_2, mm Hg	
Subject	100% O_2	90% O_2-10% CO_2	100% O_2	90% O_2-10% CO_2	100% O_2	90% O_2-10% CO_2
R	14.9	18.2	92.6	91.8	31.9	34.9
S	17.5	21.1	90.6	90.5	37.2	38.1
H	10.3	12.2	92.1	91.8	37.8	37.8
Mean	14.2	17.2	91.8	91.4	35.6	36.9
Δ		+3.0		—0.4		+1.3

Results. Average results for each series are presented in Table I. In none of the series was there any significant increase in altitude ceiling as a result of addition of carbon dioxide to the experimental mixture.

In series A, the substitution of 20% nitrogen for oxygen resulted in a slight but probably real decrease in ceiling.

In series B, the performance was definitely worse in both carbon dioxide mixtures than in 100% oxygen, and probably worse in the higher than in the lower concentration of carbon dioxide. On the other hand, in series C where the mice spent 10 minutes at 30,000 feet in the experimental mixture, instead of only 3 minutes as in series B, approximately the same altitudes were reached in 10.5% carbon dioxide as in pure oxygen. In series D, which resembles series A in that 5-minute stops were made at 2,000-foot intervals above 40,000 feet, performance was definitely better in 100% oxygen.

In view of these findings, it may be concluded definitely that mice gain no benefit, in terms of altitude ceiling, from the substitution of carbon dioxide for oxygen with the patterns employed in these experiments. In some cases, a definite harmful effect (decreased ceiling with added carbon dioxide) has been demonstrated.

Differences in the average ceiling altitude attained in a given gas mixture in the several series may be due to:

(1) Differences in altitude tolerance of individual mice; (2) Slight temperature difference between series A and the other series;

(3) Variations in the adaptation of circulatory and other mechanisms resulting from different patterns (rates) of ascent.[9]

Experiments on Human Subjects. Each of 3 male subjects was taken to a simulated altitude of 40,000 feet wearing an ear oximeter (Millikan compensated circuit) and inspiring 100% oxygen through a gas meter. Measurements of the ventilation and oximeter readings were made during a 15-minute of whether any advantage is gained by adding CO_2 to pure oxygen at high altitudes. For example, Humm *et al.*[5] and Garasenko[6] seem to find favorable effects. On the other hand, Johnson *et al.*[7] and Himwich *et al.*[8] are unable to find any benefit. The data reported below confirm the latter point of view.

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[9] Hiestand, W. A., and Miller, H. R., *Am. J. Physiol.*, 1944, **142**, 310.

Experiments on Mice. Four series of mice maintained on a stock diet were exposed in an evacuated chamber (a vacuum desiccator) to simulated altitudes in excess of 40,000 feet in various gas mixtures. Before the experiments were begun, the mice were conditioned individually to sit on a horizontal cross bar, ½ inch in diameter, which extended across the experimental chamber 3 inches above the floor. The latter consisted of a double metal grill which was connected to the secondary coil of an inductorium. When a mouse was on the grill, the operator could shock him at will by depressing a key in the primary circuit. After a few trials, the mice learned to seek safety on the cross bar, and would voluntarily take position there whenever they were put into the chamber. It was thus possible to use as an end point in the experiments the altitude at which a mouse was no longer able to maintain himself on the cross bar. In this way there was established a fairly definite criterion which did not depend on the judgment of the experimenter or place too great a strain on the endurance of the mice. After proper conditioning each mouse was exposed to altitude during a number of runs in each of the several gas mixtures.

The procedure was varied in the 4 series, as follows:

Series A. Flushout with 100% oxygen at ground level for 3 minutes; ascent to 30,000 feet in 1½ min.; flushout with experimental mixture for 5 min.

Ascent to 40,000 feet in ½ min.; stay 5 min.

Further ascent in 2000-foot stages, with 5 min. at each altitude, until ceiling was reached.

Temperature varied from 22-25°C.

Three mixtures were used: 100% oxygen (April 29 to May 5), 21.7% CO_2 in O_2 (May 8 to May 20); 20% N in O_2 (May 28 to June 13).

Series B. Flushout with 100% oxygen at ground level for 2 min.; ascent to 30,000 feet in 1½ min.; flushout with test mixture for 3 min.; continuous ascent to ceiling altitude at rate of *ca.* 6,000 ft. per min.; run in constant temperature room at 20-21°C. Three mixtures were used: 100% O_2, and 21.7% CO_2 in oxygen, on alternate days between July 10 and July 28; 10.5% CO_2 in oxygen (August 8 to August 15).

Series C. Procedure as in Series B, except that the chamber was flushed out with the test mixture at 30,000 ft. for 10 min. Two mixtures were used: 10.5% CO_2 in oxygen, and 100% oxygen, on alternate days between August 15 and August 28.

Series D. Same procedure as in Series A, except that temperature was between 20-21°C. Two mixtures were used in random succession: 10.5% CO_2 in oxygen and 100% oxygen.

In a given series, the same mice were used for all the different conditions tested. Some mice were used in more than one series. Each mouse was exposed no oftener than once a day, and usually less frequently. Only one mouse was tested in each run.

The volume of the experimental chamber was approximately 5 liters. Gas flow through the chamber was continuous whenever the altitude was stationary, and amounted to about 9 liters per minute at ground level, 2.5 liters (measured at 760 mm Hg) at 30,000 feet, 1.5 liters (at 760 mm Hg) at 40,000 feet, and 1.0 liter (at 760 mm Hg) at 50,000 feet. Inflow of gas was stopped in order to make an ascent. Altitudes were read with a mercury manometer. In the later experiments, the circuit was checked frequently for leaks, which were found to be negligible.

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[5] Humm, F. D., Liberman, A. M., and Nims, L. F., C.M.R. Report No. 335, July 18, 1944.

[6] Garasenko, V. M., *Am. Rev. Sov. Med.*, 1945, **2**, 119.

[7] Johnson, A. E., Eckman, M., Rumsey, C., and Barach, A. L., *J. Av. Med.*, 1942, **13**, 130.

[8] Himwich, H., Fazekas, J., Herrlich, H., Johnson, A. E., and Barach, A. L., *J. Av. Med.*, 1942, **13**, 177.

ponents.[5]

The work reported herein describes a study of the Newcastle disease (NCD) virus and immune serum which served as a convenient working model for the evaluation of such a test with avian serum. The hemagglutination-inhibition test was conducted simultaneously to provide a rough estimate of the reliability of the *indirect* complement-fixation results. Since chickens are known to possess an excellent capacity for antibody production, and their serums have been used for the study of various infections (influenza, psittacosis, etc.), a description of a simplified *indirect* complement-fixation test may prove of use to other investigators.

Materials and Methods. NCD immune avian serums. Serum samples were collected from chickens previously vaccinated against, or recovered from, Newcastle disease. *NCD immune guinea pig serum.* A group of guinea pigs received weekly subcutaneous injections of 0.5 ml each for 4 consecutive weeks of the antigen described below, and were bled 1 week after the final inoculation. Before pooling, the individual serum specimens were tested for complement-fixing activity by the *direct* method and only those showing titers of 1:128 or higher were included in the pool. All serum specimens were stored at 4°C.

NCD antigen. Allantoic fluids harvested from infected 11-day-old chick embryos were pooled and the virus concentrated by Sharples centrifugation. The sedimented virus was washed with 0.1 *M* phosphate buffer (pH 7.0) and resuspended in one-half the original volume of buffered saline solution[6] containing 0.05% formalin. A single lot of antigen was used in the complement-fixation and hemagglutination-inhibition tests described below.

Indirect Complement-Fixation Test. Prior to use, all serums were inactivated at 56°C for 30 minutes. Physiological salt solution was added as a diluent for all test components.

In the antigen-immune guinea pig serum titrations and in the tests proper, antigen, immune chicken serum and complement were incubated at 4 to 6°C for 2 hours. Immune guinea pig serum was then added and the tubes returned to the cold room for an additional 16- to 18-hour period (overnight). Sensitized sheep cells were added and hemolytic readings were made after an incubation period of 30 minutes at 37°C. All test components were employed in 0.2 ml amounts, thus making the total volume of 1.2 ml in all titrations and tests.

Sheep Cells. Defibrinated sheep erythrocytes were washed by centrifugation in saline solution, standardized spectrophotometrically, and adjusted to a concentration of 2%.[7]

Amboceptor. Commercial antisheep hemolysin was titrated in the usual manner and 2 units were used in all titrations and tests. A sensitized sheep cell suspension was prepared by mixing equal volumes of 2% cells and amboceptor and allowing the mixture to stand for 15 minutes at room temperature prior to use.

Complement. Serums of normal guinea pigs were pooled, 2 ml amounts were sealed in pyrex ampoules, and stored in the CO_2 icebox. Complement activity remained constant for at least 3 weeks.

For titration, amounts ranging from 0.08 to 0.22 ml (in increments of 0.02 ml) of a 1:30 dilution of complement were mixed with 0.2 ml of antigen (containing the optimal reacting amount) and with saline solution to bring the volumes to 0.8 ml. These mixtures were incubated for 1 hour at 37°C. Four-tenths ml sensitized cells were then added and hemolytic readings were made at the end of an incubation period of 1 hour at 37°C. The smallest amount of complement giving complete hemolysis was taken as 1 unit.

Preliminary titrations of NCD antigen and homologous immune guinea pig serum were carried out by the *direct* complement-fixation method using 2 units of complement. However, in the *indirect* tests, involving the deterperiod, and near the end of this interval an end-expiratory alveolar sample was secured. The inspired gas was then changed to a mixture of 10% CO_2 and 90% O_2, and measurements were continued for another 15 minutes at the end of which another alveolar sample was taken.

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[5] Wadsworth, A. B., Standard Methods of the Division of Laboratories and Research, New York State Department of Health, Albany, N. Y., Williams and Wilkins, Baltimore, Md., 1947, 3rd ed., pp. 361-465.

[6] Eagle, H., The Diagnosis of Syphilis, Mosby and Co., St. Louis, Mo., 1938.

[7] Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, 53, 37.

Results are summarized in Table II. Substitution of the CO_2-O_2 mixture for pure oxygen produced a rise of 1.3 mm in the alveolar pCO_2 (with lowering of the alveolar pO_2 by the same amount) and a drop of 0.4% in the saturation as determined from the oximeter. The change in % saturation is within the error of the instrument, but the expected change for the change in alveolar gas tensions is about 1%. The minute volume of ventilation was increased by 3 liters per minute or about 21%. These data indicate that no favorable physiological changes occurred from the addition of 10% CO_2 (9.4 mm) to the inspired gas at 40,000 feet. One cannot argue from this that it would not be advantageous to add CO_2 under some conditions, but it must be kept in mind that any rise in alveolar pCO_2 is always accompanied by an equivalent drop in alveolar pO_2 when nitrogen is absent. Probably the only condition in which substitution of a CO_2-O_2 mixture for pure O_2 might be advantageous is that of severe hypocapnia accompanied by a relatively mild hypoxia. In such a case the improvement due to the decreased hypocapnia might be greater than the impairment due to the increased anoxia.[10]

Summary. 1. The addition of 10.5% or 21.7% carbon dioxide to oxygen does not improve the altitude ceiling of mice.

2. Mice taken to altitude in 100% oxygen, with continuous ascent from 30,000 feet at a rate of about 6,000 feet per minute, collapse at a lower ceiling than mice which ascend from 40,000 feet in 2,000-foot steps with 5-minute stops at each stage.

3. In experiments on human subjects no favorable physiological effects were observed when 10% CO_2 was added to the inspired oxygen at 40,000 ft.

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[10] Otis, A. B., Rahn, H., Epstein, M. A., and Fenn, W. O., *Am. J. Physiol.*, 1946, **146**, 207.

16968

Simplified Indirect Complement-Fixation Test Applied to Newcastle Disease Immune Avian Serum.

DON M. WOLFE, LOTTIE KORNFELD, AND FLOYD S. MARKHAM.
(Introduced by Herald R. Cox.)

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The investigation in this laboratory of an avian disease presented a problem of accurate specific diagnosis. Because antigens suitable for the performance of the customary agglutination and precipitin tests were not available, attention was turned to the complement-fixation reaction. The development of the *indirect* method by Rice[1,2,3] made this approach more feasible since this technic apparently overcomes the difficulties which have prevented the use of the regular, or *direct*, method on avian serums.[4]

It also seemed worthwhile to attempt to simplify the *indirect* technic by using the common "serum or antigen-dilution" method and the observation of 100% hemolysis rather than the more detailed quantitative method for the standardization of all test com-

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[1] Rice, C. E., *J. Immunol.*, 1947, **59**, 365.

[2] Rice, C. E., *Canadian J. Comp. Med.*, 1948, **12**, 130.

[3] Rice, C. E., *J. Immunol.*, 1948, **60**, 11.

[4] Rice, C. E., *Canadian J. Comp. Med.*, 1947, **11**, 236.

TABLE II.
Results Obtained by the Indirect Complement-Fixation Test of 59 Newcastle Disease Immune Chicken Serums Compared with Hemagglutination-Inhibition Test.*

Indirect complement fixation test	Hemagglutination-inhibition test									
	0	1:16	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
0	3	1		2		1				
1:2			2							
1:8			2	3	4	2				
1:16			1	2	6	2				
1:32				1	2	4	2			
1:64				1	1	1	1			
1:128					1	2	1			
1:256					3		1	3	1	
1:512								1		
1:2048									1	1

* The numbers in the body of the table refer to the number of serums giving the endpoint titers indicated.

and No. 3. Thus, set No. 1 represented the *indirect* test, No. 2 served as the *direct* test, and No. 3 as the control for anticomplementary activity of the test serum. Additional controls were included daily for hemolytic activity of antigen, immune guinea pig serum, and antigen plus immune guinea pig serum, as well as the titration of a standard NCD immune chicken serum.

The titer of the test serum was expressed in terms of the highest dilution which inhibited fixation of complement by exhibiting ++ hemolysis or more.

Hemagglutination-Inhibition Test. Titration of Antigen. Serial twofold dilutions of virus were prepared in buffered saline solution, pH 7.1.[6] To 0.5 ml amounts of each dilution was added an equal volume of 0.25% chicken red blood cells and the mixture incubated at room temperature for 1 hour. The highest final dilution* of virus giving complete agglutination of the red cells was designated as 1 virus unit.

Test. On the basis of the above titration, a dilution of antigen containing 16 units per ml was made and used as diluent in the preparation of twofold dilutions of the test serum. To 0.5 ml amounts of the serum-virus mixtures was added an equal volume of 0.25% chicken red blood cells. Tests were read after an incubation period of 1 hour at room temperature. The endpoint of serum-inhibition was that final dilution* of serum which completely inhibited agglutination, as indicated by the flowing of the red cell deposit to a degree comparable to the red cell saline control when the test tube rack was tilted and held at approximately 70 degrees from horizontal.

Discussion of Results. The data presented in Table II show that a good, general agreement exists between the titers obtained by the *indirect* complement-fixation and by the hemagglutination-inhibition tests. No anticomplimentary serums were encountered. Four serums were negative when examined by the *indirect* complement-fixation test, yet gave positive results ranging from 1:16 to 1:512 with the other method. Nevertheless, those serums which exhibited high titers by one method also did with the other. The same holds true for specimens of intermediate and low antibody titer.

No effort was made to determine whether or not the two tests were measuring the same serum component. It is quite possible that different antibodies are involved, hence too much emphasis should not be placed on the observed agreement of test results until further information has been obtained.

However, the results of this study suggest the possibility that the described simplified *indirect* complement-fixation test should pro-

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* This term refers to the dilution of the original amount of a particular component in terms of its subsequent dilution by the addition of other components. Thus, an *initial* serum dilution of 1:8, in this case, has a *final* dilution of 1:16.

TABLE I.
A Protocol Demonstrating the Method for Determining the Optimal Proportions of Antigen and Immune Guinea Pig Serum to Be Used in the Indirect *Complement Fixation Procedure.*

Dilutions of antigen	Dilutions of immune guinea pig serum	Dilutions of immune chicken serum								
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control*
1:8	1:70	—	—	—	—	++	++++	++++	++++	++++
	1:80	—	—	—	—	±	+++	++++	++++	++++
	1:90	—	—	—	—	—	+++	++++	++++	++++
	1:100	—	—	—	—	—	—	++	++++	++++
	1:110	—	—	—	—	—	+	+	+++	++
	1:120	—	—	—	—	—	—	+	+	++
	1:130	—	—	—	—	—	±	+	++	+++
1:16	1:20	—	—	—	—	+	++	++++	++++	++++
	1:30	—	—	—	—	—	++	+++	+++	++++
	1:40	—	—	—	—	—	+	+	+++	+++
	1:50	—	—	—	—	—	—	+	+++	+++
	1:60	—	—	—	—	—	—	++	++	++

* Immune chicken serum replaced by saline solution.

mination of optimal amounts of antigen and immune guinea pig serum to be used in the titration of an immune chicken serum, it was found that 1.5 units of complement gave more uniform and consistent results than the larger quantity. This amount is approximately equivalent to the three 50% units employed by Rice.[2,3]

Titration of Antigen. A preliminary antigen titer of 1:8 was obtained by the simultaneous "cross titration" of both antigen and immune guinea pig serum by the *direct* method. It will be seen that this titration furnished a fairly reliable basic titer for use in the *indirect* method. The titration by the latter method is described in the following section.

Titration of Immune Guinea Pig Serum. That the antigen and known immune serum (guinea pig) be present in proportions which would permit maximal inhibition of fixation by the test serum (chicken), has been stressed by Rice.[1,2] A simplified procedure for the titration of these components was developed by simultaneously testing varying amounts of antigen and immune guinea pig serum in the presence of varying amounts of a known immune chicken serum, as illustrated in Table I.

The amounts of antigen and immune guinea pig serum giving complete inhibition of hemolysis in the control tube (*i.e.,* in the *absence* of immune chicken serum), and complete hemolysis in the presence of the smallest quantity of immune chicken serum, were selected as the optimal amounts to be used in subsequent tests. In this case an antigen dilution of 1:8 and an immune guinea pig serum dilution of 1:100 were found to be the properly reacting quantities of these components.

Test. The test technic was similar to that described by Rice[2] in which serial twofold dilutions of the suspected immune chicken serum were prepared in triplicate. Antigen was added to sets No. 1 and No. 2, saline solution to set No. 3. All tubes then received complement. After the preliminary cold incubation, immune guinea pig serum was added to set No. 1 and saline solution to sets No. 2

TABLE I.
Amino Acids in Salmin and Clupein (free base).

	Salmin				Clupein	
Amino acid	Block and Bolling[1] and Authors %	Kossel and Dakin (cf. [3]) %	Taylor[12] %	Tristram[2] %	Authors %	Kossel[3] %
Arginine	88.4	87.4	91.7	85.2	87.3*	88.0
,,					86.8†	
Proline	7.9	11.0	10.8	5.8	8.2†	
,,	8.6†					
Serine	7.0	7.8	8.7	9.1	3.4	
Valine	4.1	4.3	5.3	3.1	3.6	
Alanine	1.5			1.1	4.7	
Isoleucine	1.2			1.6	1.0	
Leucine	0†				0†	
Glycine	3.3			2.9	0	
Threonine	0				1.9	
Nitrogen	31.52	31.52			31.68	

* Kossel flavianate method.
† Microbiological method.

salmon or herring milt obtained through the kindness of Mr. Dudley Cole, Victoria, B.C., during the past 4 years. The protamine sulfate preparations were devoid of contaminating material as evidenced by negative Molisch, Millon, Pauly, ninhydrin, and phosphorus tests. (*cf.*[1,4]). The purity of each preparation was further confirmed by the use of two-dimensional paper chromatograms.[5]

2. *Amino Acid Analysis.* Arginine was determined by precipitation as the monoflavianate (*cf.*[4]) and microbiologically.[6] Proline was estimated by Guest's pyrrole method (*cf.* [4]) and by the microbiological procedures of Henderson and Snell[7] or of Dunn *et al.*[8] Serine and threonine were determined by periodate oxidation (*cf.* [4]). Glycine and alanine were determined by oxidation with ninhydrin to HCHO and CH_3CHO respectively (*cf.* [4,9,10]) Leucine, isoleucine, and valine were estimated by microbiological methods[4,11,12]. Leucine does not appear to be present in either of these protamines.

Results and Discussion. Our analytical results, as well as those of Kossel and Dakin (*cf.* [3]), Taylor,[13] and Tristram,[2] are given in Table I.

In contrast to the results of Felix and Mager,[14] there is no evidence for the presence of hydroxyproline in our samples of clupein. Because salmin contains no demonstrable amino or imino nitrogen or free carboxyl groups, Frankel-Conrat and Olcott[15] suggested that salmin lacks an end-group and that it may have a cyclic structure similar to those suggested for gramicidin and tyrocidine. Waldschmidt-Leitz *et al.*[16] reported that the vide a useful tool in investigations involving the examination of serums of certain species in which the *direct* complement-fixation procedures are not feasible.

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[5] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

[6] Stokes, J. L., *et al.*, *J. Biol. Chem.*, 1945, **160**, 35.

[7] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.

[8] Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 703.

[9] Alexander, B., Landwehr, G., and Seligman, A. M., *J. Biol. Chem.*, 1945, **160**, 51.

[10] Alexander, B., and Seligman, A. M., *J. Biol. Chem.*, 1945, **159**, 9.

[11] Shankman, S., *J. Biol. Chem.*, 1943, **150**, 305.

[12] McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, 1944, **152**, 83.

[13] Taylor, A. E., *J. Biol. Chem.*, 1908-09, **5**, 389.

[14] Felix, K., and Mager, A., *Zeit. physiol. Chem.*, 1937, **249**, 112.

[15] Frankel-Conrat, H., and Olcott, H. S., *Fed. Proc.*, 1947, **6**, 253.

[16] Waldschmidt-Leitz, E., Ziegler, F., Schäffner, A., and Weil. L., *Z. physiol. Chem.*, 1931, **197**, 219.

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16969

Preparation and Amino Acid Composition of Salmin and Clupein.

RICHARD J. BLOCK, DIANA BOLLING, HERMAN GERSHON, AND HERBERT A. SOBER.
From the Department of Physiology and Biochemistry, New York Medical College, New York.

Salmin, on acid hydrolysis, was shown to yield arginine, proline, serine, valine, alanine, and isoleucine.[1] This observation was confirmed and extended by Tristram[2] who also identified glycine. This paper confirms the presence of glycine in salmin and also indicates that clupein, from *Clupea pallasii*, differs from salmin in that it contains threonine but no glycine.

1. *Preparation.* The procedure to be described gives approximately 10 times the yield of protamine from frozen milt than was obtained by any of the older methods.[3] One thousand grams of frozen milt are thawed and rinsed with cold tap water, mixed with an equal volume of a 1% solution of citric acid, finely ground, and 2.5 liters of 1% of citric acid are added. After standing in the refrigerator over night, the insoluble material is removed by centrifugation and the residue is washed with citric acid. This precipitate is suspended in 4 liters of 0.2 *N* HCl for 18-24 hours at room temperature with occasional mixing. The precipitate is removed and washed with 0.2 *N* HCl. The combined filtrate and washings are heated to boiling and adjusted to pH 7-8 with dilute ammonia. The resulting precipitate is removed by centrifugation and ammonia is added to the filtrate, at 30°C, to bring the pH to 9.4-9.6. The protein precipitate is removed by filtration. The contaminating proteins may also be removed by precipitation with 14% ammonia at room temperature at pH 10.4-10.6. In contrast to the first method, the histones (?) so prepared are completely soluble in 0.2 *N* HCl.

The filtrate is cooled to 12-15°C. Aliquots of the cold solution are removed and graded quantities of freshly prepared 33% of HPO_3 are added. The oily precipitates of protamine metaphosphate are thrown down by centrifuging and are washed with cold water. The quantity of HPO_3 required for optimal precipitation is thus ascertained.

The calculated quantity of 33% HPO_3 is then added slowly with stirring to the main protamine solution and the resulting oil is removed and washed with cold water until the washings are free from ammonia. The protamine metaphosphate precipitate is dissolved in a minimum quantity of hot *N* sulfuric acid. The solution is boiled until a small aliquot no longer gives a white cloud when cooled below 5°C, *i.e.*, when the metaphosphate has been completely converted to the sulfate but no longer. Protamine sulfate is precipitated and dried by the addition of alcohol or acetone. The *yield is 2.0-2.5%* of the weight of the original milt.

More than 30 preparations of salmin sulfate or hydrochloride and 8 preparations of clupein sulfate have been prepared from samples of

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More than 30 preparations of salmin sulfate or hydrochloride and 8 preparations of clupein sulfate have been prepared from samples of with an iced pipette in a test tube and mixed with 4 cc 10% trichloracetic acid. After 30 minutes 8 cc 5% trichloracetic acid was added and after inverting the tube twice the contents were filtered. The filtrate was kept in the ice box and 2 cc were used for each determination. The amount of adrenalin-like compounds was expressed as the adrenalin chloride equivalent (in gammas/100 cc of blood).

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[2] Tristram, G. R., *Nature*, 1947, **160**, 637.

[3] Kossel, A., Protamine und Histone; Leipsig, Wien, 1929.

Electronarcosis was produced by applying a 60-cycle alternating current to the brain through electrodes placed on both sides of the head directly behind the eyes. Electronarcosis was started with a relatively high current 150-200 mA, which was continued for 30 seconds. The current was then dropped to 25-40 mA after which respiration was reestablished. This current was increased or decreased as needed to maintain a quiet electronarcosis.[9] The total duration of electronarcosis in the present experiments was limited to 5 minutes. Rabbits were used exclusively.

Results. In order to establish a control two blood samples were taken with a 10-minute interval before electronarcosis. In 33 experiments the mean of the level of adrenalin-like compounds in the first of these samples was 21 gamma/100 cc; the individual values varying between 17 and 25 gamma/100 cc. The difference between the two control values supplies information about the variability of the determination and about changes in the concentration of the adrenalin-like compounds during a 10-minute period. The mean difference in these 33 experiments was 0.03 gamma/cc, the largest difference was 3 gamma/cc. Using a different modification of the Whitehorn procedure, Kobro[10] obtained comparable values for the adrenalin-like compounds in rabbit's blood.

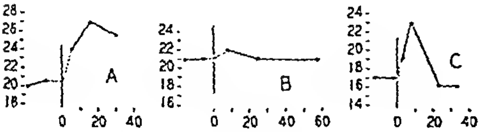

FIG. 1.

The level of adrenalin-like compounds (in gammas per 100 cc) *is plotted against time in minutes.* The vertical line in A indicates the beginning of a 5-minute electronarcosis, in B the same in a curarized and vagotomized preparation, and in C the beginning of a 2½-minute asphyxiation in a curarized preparation.

In agreement with the observations on patients the level of adrenalin-like compounds rose during electronarcosis (Fig. 1, A). In 9 experiments the mean of the maximal rise in the level was 5 gamma/100 cc. The mean difference between the two control determinations in these 9 experiments was 0.2 gamma/100 cc. Statistical treatment of these data shows that the increase of the level due to electronarcosis is significant at the 5% level ($t = 2.7$).

As shown in Fig. 1, A, the level of adrenalin-like compounds has started to decline again 30 minutes after the end of electronarcosis. It is of interest to note that Kobro[10] observed a fall of the level 30 minutes after an injection of adrenalin.

The initial stage of electronarcosis is characterized by a modetately severe asphyxiation of the animal and by convulsions. Both concomitants can be prevented by the administration of Intocostrin (0.5-0.8 cc/kg) combined with artificial respiration. Furthermore such preparations were doubly vagotomized to prevent the cardiac arrest which usually occurs in the beginning of electronarcosis[9] and which might cause a short systemic asphyxiation. The effect of electronarcosis on the level of adrenalin-like compounds in such preparations was variable, but in general an unusually small rise of the level was found. The results of one of these experiments are given in Fig. 1, B.

Since there was some indication that either convulsive activity or asphyxiation (or both) might cause the increase of adrenlin-like compounds during electronarcosis, the effect of asphyxiation alone was examined. In curarized preparations the trachea was clamped for a total of 2½ minutes (usually with a short interval when the heart action became weak). This caused a rise in the level of the compounds which was of the same order as the increase observed after imino group of proline is one terminal group of salmin and the carboxyl of arginine the other. Tristram[2] mentions unpublished results of Porter using the dinitrofluorobenzene method as strong evidence that proline is the end-group of salmin. This may account for the absence of the ninhydrin color of these protamines.

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[9] Frostig, J. P., van Harreveld, A., Reznick, S., Tyler, D. B., and Wiersma, C. A. G., *Arch. Neurol.*, 1944, **51**, 232.

[10] Kobro, M., *Acta Med. Scand.*, 1946, **125**, 49.

The data obtained in this study indicate that the ratio of arginine to monoamino acids in both salmin and clupein is somewhat higher (*cf.* [1]) than the 2:1 ratio postulated by Kossel.[3]

The question of homogeniety of salmin was discussed in a previous publication.[1] A similar situation holds with respect to clupein. Since that time, numerous unsuccessful attempts to fractionate salmin and clupein have been made from 90% aqueous isopropanol at 90°C, 25°C, and 0°C, and by paper chromatography using water-saturated phenol, lutidine-water-alcohol, water alone, 0.1 *N* H_2SO_4 and other solvents. The dinitrophenyl derivatives of the protamines also failed to yield more than one spot on paper chromatograms using various solvents. Although these preparations may be chemically inhomogeneous, their overall physical properties must be rather similar due to the preponderance of arginine.

Summary. A simplified procedure for the preparation of salmin and clupein is described. The amino acid content of clupein as well as salmin has been estimated.

16970

Effect of Electronarcosis on Level of "Adrenalin-like" Compounds in Blood.

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A chemical method for the quantitative determination of adrenalin was devised by Whitehorn.[1] This procedure was modified by Shaw[2,3] who showed that the method is not specific for this compound, and that a number of substances related to adrenalin give the same reaction. Shaw's method was applied to blood by Tietz, Dornheggen and Goldman,[4] Raab,[5] Bloor,[6] and others. It became apparent that the material thus estimated is not adrenalin alone, but that other compounds determined by Shaw's procedure are present in the blood.

Previous observations showed that the level of these "adrenaline-like" compounds is increased in the blood of patients during insulin shock.[7] It was also found to be increased during electronarcosis and electroshock treatment in humans.[8] In the present paper the effect of electronarcosis on the level of these compounds was investigated experimentally.

Methods. The technic used for the estimation of adrenalin-like compounds in blood has been reported before.[7] In order to limit the total amount of blood taken from the animal the method was adapted in the following way to the use of about 2 cc per determination. Blood was taken from the jugular vein which had been exposed previously under local anesthesia. A little more than 2 cc of blood was drawn from the vein in an iced syringe and squirted immediately into a small tube cooled in ice. Of this, 2 cc was measured

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[1] Whitehorn, C., *J. Biol. Chem.*, 1935, **108**, 633.

[2] Shaw, F. H., *Biochem. J.*, 1938, **32**, 19.

[3] Shaw, F. H., *Australian J. Exp. Biol. and Med. Sc.*, 1946, **24**, 53.

[4] Tietz, E. B., Dornheggen, H., and Goldman, D., *Endocrinology*, 1940, **26**, 641.

[5] Raab, W., *Endocrinology*, 1941, **28**, 325.

[6] Bloor, W. R., *J. Biol. Chem.*, 1939, **128**, ix.

[7] Tietz, E. B., and Birnbaum, S. M., *Am. J. Psych.*, 1942, **99**, 75.

[8] Tietz, E. B., unpublished data.

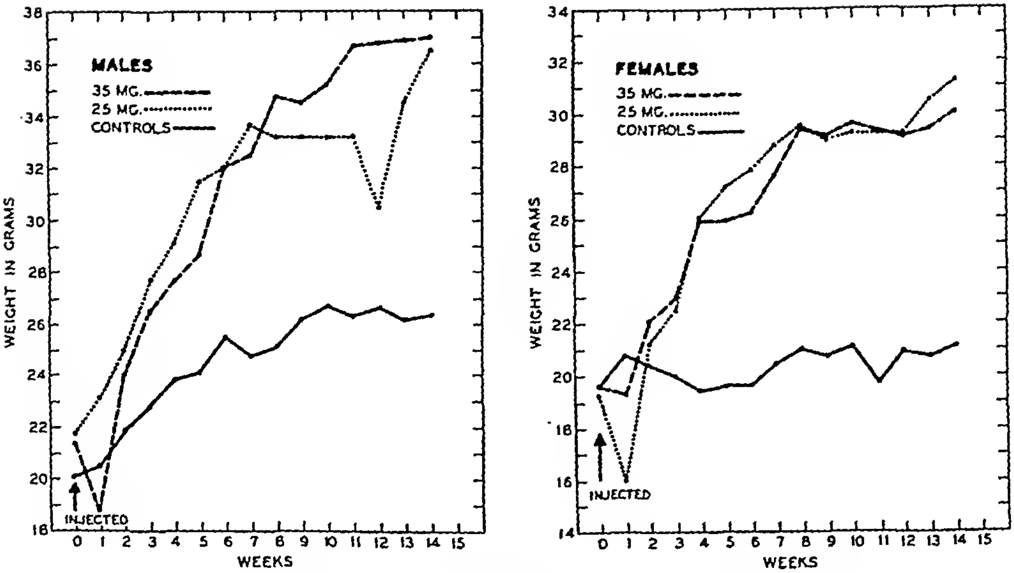

FIG. 1.

Average weights of male and female mice injected with goldthioglucose (25 and 35 mg), compared with controls.

of goldthioglucose intraperitoneally. Thirty males and 30 females were given 35 mg of the compound, and 5 males and 5 females were given 25 mg of the compound. Of the animals injected with 35 mg of goldthioglucose, 15 males and 24 females survived. Of the animals injected with 25 mg of the compound, 4 males and 4 females survived. The average weight of these survivors and of the controls is represented in Fig. 1. The differences in weight gains between treated and untreated animals were statistically significant in both males and females.

In another experiment, some of the control mice were injected with 0.5 cc of sesame oil. This was done to exclude the possibility that the oil of sesame used as the vehicle for goldthioglucose may have an effect on body weight. Out of a group of 25 male mice, 15 were injected intraperitoneally with 25 mg in 0.25 sesame oil. Seven of these survived. Four animals received 0.5 cc sesame oil intraperitoneally, and 5 animals were left untreated. Fig. 2 shows the individual weights of these animals at weekly intervals. The difference in weights of untreated controls and those injected with sesame oil were not statistically significant, while the animals given goldthioglucose in sesame oil showed significantly greater weight gains. An identical experiment with female mice showed similar results.

In order to test the possible influence of the route of administration, one group of mice was given a single dose of 25 mg of goldthioglucose intramuscularly. Because of the small muscle mass available, the dose was split and 12.5 mg of goldthioglucose was given in each thigh. The weight curves of these animals were in all respects similar to those of the intraperitoneally injected animals. Weight increases in the injected group were again significantly higher than in the controls.

In all these experiments only one-third to one-half of the injected animals showed unusual weight gains. This is well shown in Fig. 2, in which the individual weights of animals surviving the injection of 25 mg goldthioglucose are recorded at weekly intervals. In several experiments animals that failed to show significant weight gains by the eighth week were given another 25- or 35-mg dose of goldthioglucose. Significant weight gains were subsequently observed in a number of re-injected animals.

Animals injected with 12 mg of goldthioelectronarcosis. Fig. 1, C, shows the results of such an experiment. The mean increase of 4 experiments was 6 gamma/100 cc. In the blood of animals killed by asphyxiation the level rises even more, up to 38 gamma/100 cc has been observed.

Discussion and Summary. There is reason to believe that when asphyxiation is prevented electronarcosis causes only small increases of the level of adrenalin-like compounds in blood. It had been found previously[11] when one of two rabbits connected by a double carotid-jugular anastomosis was given an asphyxia-free electronarcosis, that the blood pressure in the other showed a slight increase. This was believed to be due to a release of adrenalin, which in the present experiments may cause the slight rise in the level of "adrenalin-like" compounds during asphyxia-free electronarcosis. It seems likely that the much larger increase of these compounds which is always present in animals subjected to electronarcosis without further precautions, is caused by asphyxiation.

Kobro[12] did not observe any effect of respiratory impairment (letting the animal breathe through a tightly woven cloth) on the adrenalin level. The impairment employed in the present experiments, though of shorter duration, was more severe (clamping of the trachea), which may account for the difference in the results obtained.

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[11] van Harreveld, A., and Dandliker, W. B., PROC. SOC. EXP. BIOL. AND MED., 1945, 60, 391.

[12] Kobro, M., *Acta Med. Scand.*, 1946-47, 126, 97.

16971

Obesity in Albino Mice Due to Single Injections of Goldthioglucose.

GEORGE BRECHER AND SAMUEL H. WAXLER. (Introduced by K. M. Endicott.)

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During a toxicological study of goldthioglucose, it was noted that a number of albino mice receiving this compound showed a marked increase in size. This response occurred in animals which had been injected with a single dose of this drug. The present investigation was undertaken in an attempt to define the conditions under which these gains in weight occur.

Experimental. Goldthioglucose is an unstable compound of gold and thioglucose, containing 50% gold. It dissociates in the presence of water. The compound is hygroscopic and is therefore utilized as a suspension in sesame oil. The concentration of the suspension used in these experiments was 100 mg of goldthioglucose in 1 cc of the oil (supplied by Schering Corporation, Bloomfield, N.J.). The route of administration was by intraperitoneal or intramuscular injection.

Stock male and female mice were used. The animals were kept on a pellet diet, and fed *ad lib.* In some of these studies the animals were kept in individual cages and the food intake determined. All animals were weighed at weekly intervals.

The LD_{50} of goldthioglucose was 40-50 mg intraperitoneally for mice weighing 20 g. Fifty to 70% of the animals survived doses of 35 mg of goldthioglucose, and 90% survived doses of 25 mg. All animals survived the intraperitoneal injection of 12 mg of the compound. Fatalities from the administration of goldthioglucose generally occurred within the first 3 to 4 days. There was no further mortality due to the toxicity of the drug among those animals which survived one week.

In one representative experiment, animals weighing 18-23 g were given single injections

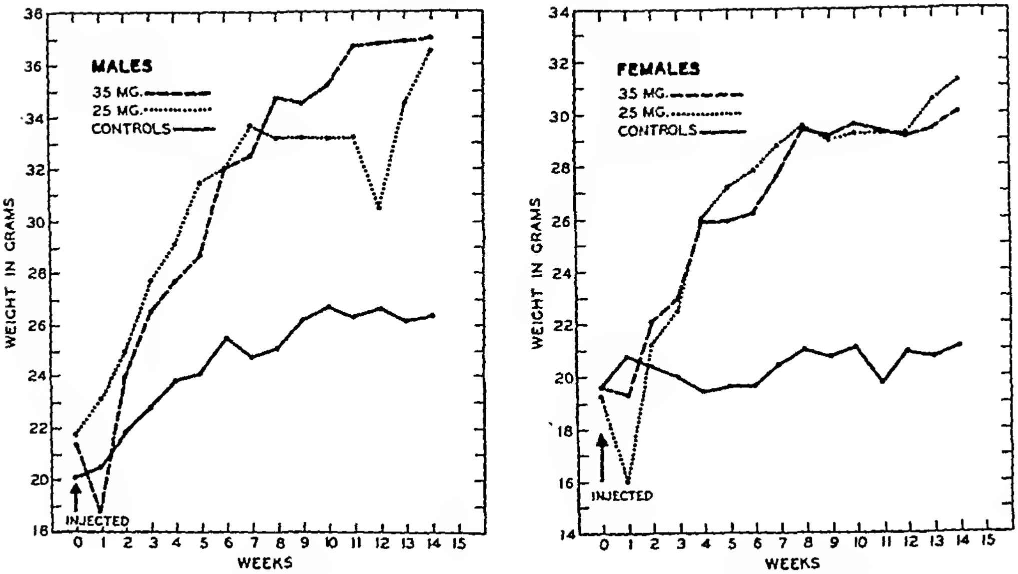

FIG. 1.
Average weights of male and female mice injected with goldthioglucose (25 and 35 mg), compared with controls.

of goldthioglucose intraperitoneally. Thirty males and 30 females were given 35 mg of the compound, and 5 males and 5 females were given 25 mg of the compound. Of the animals injected with 35 mg of goldthioglucose, 15 males and 24 females survived. Of the animals injected with 25 mg of the compound, 4 males and 4 females survived. The average weight of these survivors and of the controls is represented in Fig. 1. The differences in weight gains between treated and untreated animals were statistically significant in both males and females.

In another experiment, some of the control mice were injected with 0.5 cc of sesame oil. This was done to exclude the possibility that the oil of sesame used as the vehicle for goldthioglucose may have an effect on body weight. Out of a group of 25 male mice, 15 were injected intraperitoneally with 25 mg in 0.25 sesame oil. Seven of these survived. Four animals received 0.5 cc sesame oil intraperitoneally, and 5 animals were left untreated. Fig. 2 shows the individual weights of these animals at weekly intervals. The difference in weights of untreated controls and those injected with sesame oil were not statistically significant, while the animals given goldthioglucose in sesame oil showed significantly greater weight gains. An identical experiment with female mice showed similar results.

In order to test the possible influence of the route of administration, one group of mice was given a single dose of 25 mg of goldthioglucose intramuscularly. Because of the small muscle mass available, the dose was split and 12.5 mg of goldthioglucose was given in each thigh. The weight curves of these animals were in all respects similar to those of the intraperitoneally injected animals. Weight increases in the injected group were again significantly higher than in the controls.

In all these experiments only one-third to one-half of the injected animals showed unusual weight gains. This is well shown in Fig. 2, in which the individual weights of animals surviving the injection of 25 mg goldthioglucose are recorded at weekly intervals. In several experiments animals that failed to show significant weight gains by the eighth week were given another 25- or 35-mg dose of goldthioglucose. Significant weight gains were subsequently observed in a number of re-injected animals.

Animals injected with 12 mg of goldthio-

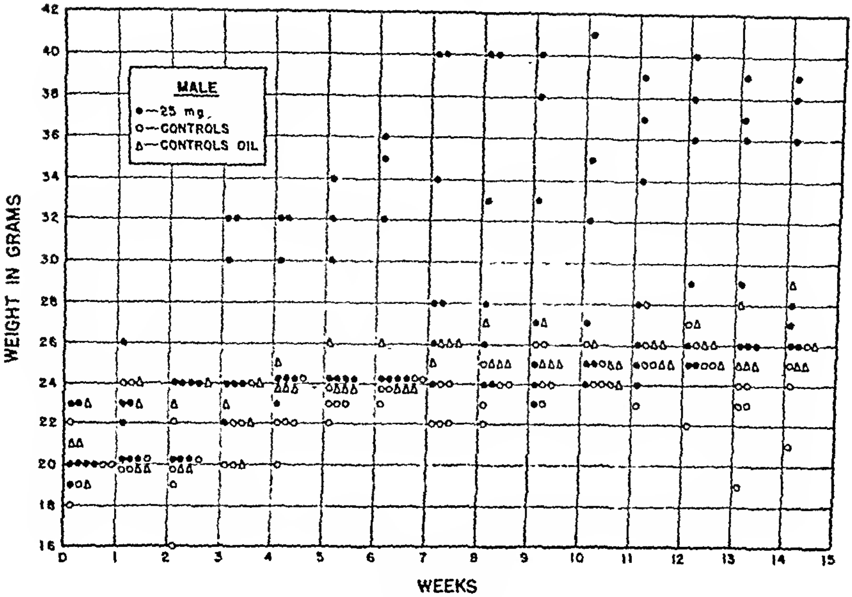

FIG. 2.

Comparison of individual weights of male mice injected with goldthioglucose, sesame oil, and controls.

glucose intraperitoneally showed no significant weight increases as compared with the control animals. Animals given injections of 5 mg of goldthioglucose intraperitoneally twice a week until a total of 150 mg was given, failed to show any significant weight gains.

All animals used in the above experiments were killed at the end of 14 weeks. There was marked increase in intra-abdominal fat and some increase in intrathoracic fat in the heavy animals. There was also a moderate increase in subcutaneous fat in the pelvic region and the region of the shoulder girdle. The viscera and the brain of both injected and control animals were examined microscopically. Mild inflammatory reactions at the site of injection and histochemically demonstrable gold in reticulo-endothial cells of various organs were present in all injected animals. There was mild to moderate centrolobular fatty infiltration of the liver in obese animals. This fatty infiltration ran roughly parallel with the weight of the animals. No other lesions were found in the liver, spleen, pancreas, kidney, adrenal, genital organs, heart, lung, lymph nodes, brain, hypophysis, thyroid, parathyroids, and adipose tissue.

Autopsy findings indicated that the abnormal amount of adipose tissue in injected mice might account for most of their weight gain. To verify this observation, the total body fat content was determined by the gravimetric method, using acid hydrolysis and ether extraction. Nitrogen, water, and ash were determined on aliquots from the same animals. Untreated controls selected at random were used for these determinations and compared with a group of injected animals showing marked weight gains. The weight of the injected animals so selected ranged from 38 to 60 g, and that of the controls from 18 to 25 g. The mean values and standard errors of the analytical determinations in the two groups are given in Table I. These analyses showed that the weight difference between injected heavy animals and non-injected controls could be primarily accounted for by the marked increase in adipose tissue in the injected animals.

Roentgenological examinations of the skeleton of obese and normal animals showed no recognizable difference in size, shape, or density of bones.

Summary. Marked weight gains were produced in stock albino mice by single injec-

TABLE I.
Total Lipids, Protein, Water, and Ash.

	Normal mice (7 animals)					Injected mice showing marked wt gains (7 animals)				
	Wt, g	Lipids g	Protein g	Water g	Ash g	Wt, g	Lipids g	Protein g	Water g	Ash g
Mean	22.1	2.42	3.65	16.2	.74	45.6	18.07	5.55	20.0	.72
Stand. error	.9	.33	.12	.7	.05	2.4	1.94	.26	.9	.09

tions of goldthioglucose. Analysis of total body lipids, protein, water, and ash showed these weight gains to be primarily due to an increase in adipose tissue. This obesity occurred in approximately one-third of the survivors of sublethal doses of goldthioglucose. Except for centrolubular fatty infiltration of the liver in obese animals, no anatomic lesions were found in the organs examined.

16972

Effect of Aminophylline on Plasma Prothrombin and Ac-Globulin in Dogs.

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Several conflicting reports have appeared in recent literature concerning the effect of the administration of methylxanthines on prothrombin activity. Field and coworkers[1] reported a hyperprothrombinemia following oral administration of these drugs to experimental animals in doses ranging from 10-400 mg/kg body wt. Scherf and Schlachman[2] also reported a shortening of prothrombin time in human subjects given therapeutic doses of aminophylline and theophylline with sodium acetate either orally or intravenously. However, a number of investigators have been unable to confirm these results. Quick[3] noted no increase in the prothrombin level of dogs and rabbits fed large doses (100-200 mg/kg) of methylxanthines. In a careful study, Holland and Gross[4] reported that methylxanthines, when given to dogs either in single intravenous doses of 25-50 mg/kg, or in daily oral doses of 20 mg/kg, produced no shortening in prothrombin time. Furthermore, single intravenous doses of 0.5 g of aminophylline failed to shorten the prothrombin time of human subjects. Several other studies[5-8] with human subjects failed to demonstrate significant changes in prothrombin time after oral or intravenous administration of aminophylline. All studies reviewed above were done by using only one-stage methods for the determination of prothrombin. Either Quick's original technique or one of its numerous modifications were utilized.

Because of recent advances in the under-

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* Aided by a grant from the United States Public Health Service.

[1] Field, J. B., Larsen, E. G., Spero, L., and Link, K. P., *J. Biol. Chem.*, 1944, **156**, 725.

[2] Scherf, D., and Schlachman, M., *Am. J. Med. Sc.*, 1946, **212**, 83.

[3] Quick, A. J., *J. Biol. Chem.*, 1945, **161**, 33.

[4] Holland, M. S., and Gross, E. G., *J. Iowa St. Med. Soc.*, 1948, **38**, 183.

[5] Breytspraak, R. W., and Greenspan, F. S., *Am. J. Med. Sc.*, 1946, **212**, 476.

[6] Poindexter, C. A., and Meyers, L., *Quart. Bull. Northwestern Univ. Med. School*, 1946, **20**, 130.

[7] Gilbert, N. C., Dey, F., and Trump, R., *J. Lab. and Clin. Med.*, 1947, **32**, 28.

[8] Blood, D. W., and Patterson, M. C., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 130.

standing of the blood clotting mechanism, and improved methods of analysis,[9-13] the problem was investigated further in this laboratory. It seemed especially important to take into account the newly recognized factor which accelerates the conversion of prothrombin to thrombin. This accelerator (Ac-globulin) is now recognized to be an integral part of the coagulation mechanism. A quantitative method for measuring its concentration has been developed.[12] It is essentially an adaptation of the two-stage method for prothrombin determination. The original two-stage method for prothrombin determination has also been modified[13] to account for variation in the activity of Ac-globulin. Using these newer techniques, we have, in this study, been able to demonstrate a definite effect of aminophylline on the coagulation mechanism in dogs.

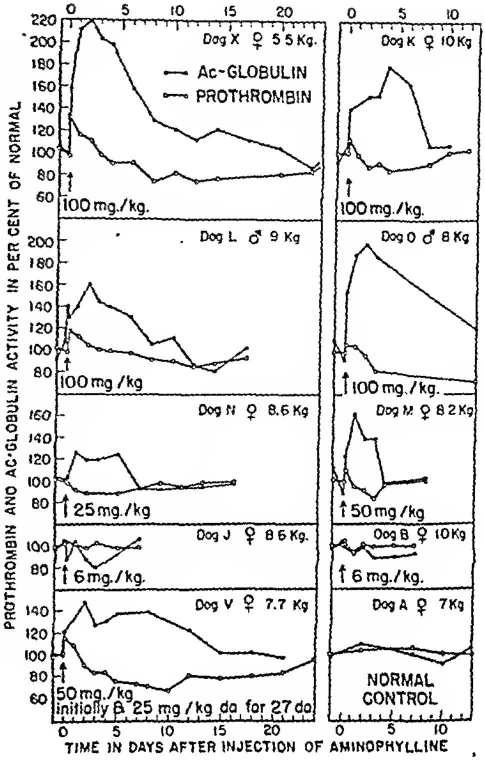

FIG. 1.

The protocol shows changes in plasma Ac-globulin and prothrombin concentration for 9 experimental animals and one typical control.

Methods. Following a control period of 2 to 4 days, aminophylline was administered intravenously in varying dosages to healthy dogs maintained on a stock diet. Blood samples were drawn 4, 24, 48, and 72 hours after administration of the drug, and subsequently at 2 to 3 day intervals until normal values were obtained. To avoid thrombin formation we observed ordinary precautions in withdrawing blood and followed largely the technique described elsewhere.[14] One part of 3.2% sodium citrate was added to 9 parts blood. The plasma was removed following centrifugation and stored in a frozen state at —20°C until determinations were performed. Serial hematocrit determinations were made at first but because no significant variations were noted, this was later discontinued. The clotting time of whole blood, using the Lee-White method, was also followed at frequent intervals.

Prothrombin activity was determined by the modified two-stage method described by Seegers,[13] and Ac-globulin activity was estimated by the method of Ware and Seegers.[12] All determinations were compared to a standard control run simultaneously, so that minor corrections could be made for variations in reagents.

Results. Fig. 1 illustrates the results obtained with 8 dogs given single, intravenous doses of aminophylline, and with one dog given multiple intravenous injections. Prothrombin and Ac-globulin activities are expressed in per cent of normal control values. Expressed in units per cc of citrated plasma, normal prothrombin activity ranged from 170 to 185; Ac-globulin activity from 185 to 230.

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[9] Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

[10] Owren, P. A., The Coagulation of Blood, Investigations on a New Clotting Factor, Oslo, 1947.

[11] Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **231**, 169.

[12] Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **172**, 699.

[13] Seegers, W. H., Blood Clotting and Allied Problems, Josiah Macy, Jr. Foundation, Feb., 1948.

[14] Fahey, J., Olwin, J. H., and Ware, A. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 491.

All animals were first observed for a control period of a week or so to establish their particular normal (100%) levels.

Following the administration of 100 mg of aminophylline per kg body wt, a small but consistent rise in prothrombin activity was noted in 4 hours, falling to normal by 24 to 48 hours (dogs X, Q, L, and K). This was followed by a drop to approximately 15 to 30% below normal, gradually returning to normal in about 2 to 3 weeks. Ac-globulin activity rose sharply in 4 hours, reached a maximum in 48 to 96 hours, and then gradually fell to normal levels. Determinations on blood drawn 2 hours after administration of aminophylline to dog L showed that a significant response was present even at this time. This characteristic pattern was less evident with smaller doses (50 and 25 mg/kg body wt). Administration of 6 mg/kg body wt (approximate therapeutic dosage in man) was without effect on either prothrombin or Ac-globulin activity (dogs B and J).

Several dogs given salts of caffeine and theobromine intravenously in large single doses (100 mg/kg body wt), were found to respond in the same characteristic manner.

Dog V, given 50 mg of aminophylline per kg body wt initially, and then daily doses of 25 mg/kg body wt for 4 weeks, demonstrated that the elevation in prothrombin activity could not be maintained by repeated injections. Increased Ac-globulin activity persisted for a longer period but returned to normal in spite of continued administration of the drug.

As a matter of interest, plasma from several of these dogs was analyzed for prothrombin activity using the one-stage method. The technique originally described was carefully followed, using 100% plasma. Deviations from the normal values of 6 to 6.5 seconds were found with the large intravenous doses of the drug. This paralleled the results obtained using the two-stage method. For example, the prothrombin time of dog L fell to 5 seconds 4 hours after administration of aminophylline, remained at 5½ seconds for 96 hours, and increased to 7 seconds before returning to normal. On the other hand, dog J showed no deviation from normal.

No correlation could be found between the Lee-White clotting time of whole blood and variations in prothrombin and Ac-globulin activities. This has also been noted by other authors and is undoubtedly due to inadequacies of the method. A modified technique has been described by Blood and Patterson[8] which is probably a considerable improvement over the method we used. These investigators consistenly obtained a very narrow range of normal values for the clotting time of whole blood.

Summary. 1. Large doses of aminophylline, administered intravenously to dogs, produced a transient elevation in prothrombin activity, followed by a late hypoprothrombinemia with return to normal in about 2 to 3 weeks. Simultaneously, a more marked and persistent rise in Ac-globulin activity occurred. Small doses of 6 mg/kg body wt were without significant effect.

2. No correlation could be found between the Lee-White whole blood clotting time and variations in prothrombin and Ac-globulin activity.

16973

Effects of Adrenoxyl on Blood Coagulation Mechanism and Vasomotor Response.

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(Introduced by J. A. Shannon.)
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Cannon and Mendenhall[1] have shown that adrenalin shortens the coagulation time of blood. Roskam, Derouaux and associates[2] studied the effect of small doses of adrenalin on the bleeding time and reported a hemostatic action following the administration of the drug.

Adrenoxyl is a relatively stable derivative of adrenalin[*] developed by Braconier and associates.[3] Adrenochrome, adrenoxime,[4,5] and adrenoxyl,[6] oxidative derivatives of adrenalin, are reported as being capable of reducing the bleeding time approximately 25%. The adrenoxyl effect is demonstrable 15 minutes after intravenous injection in rabbits and 30 minutes after injection in man. The optimal doses are 0.001 mg in the rabbit and 0.1 to 0.5 mg in man. Derouaux[6] and Bacq[7] report that the circulation in rats and rabbits remains unaltered by the above doses of Adrenoxyl. These investigators emphasize lack of any sympathomimetic effect as reflected by the absence of change in the blood pressure and pulse rate in animals following injection of the drug. Roskam and associates believe that Adrenoxyl has a vasomotor effect which is responsible for the rapid occlusion of injured vessels. On the other hand, Desfontaines[8] has found a vasodilator effect of adrenochrome in man and recommends it for the treatment of hypertension.

As a preliminary study of the pharmacological properties of Adrenoxyl in relation to the bleeding time, we followed the administration of the drug with these screening procedures: Clotting time; prothrombin time; pulse wave velocity; skin temperature; and bleeding time determination. Such procedures would yield information concerning alterations in coagulability of blood and the vasomotor response of the injured vessels.

Methods and Results. Adrenoxyl prepared by Labaz Laboratories in ampules containing 0.1 and 0.5 mg in solution, was used in this investigation.[†] The prothrombin time and bleeding time determinations were performed on rabbits of both sexes. All other studies were made on healthy men between 17 and 39 years of age.

1. *Clotting time* (Table I). Blood samples were taken from the arm veins of 6 seated individuals and drawn directly into capillary tubes by a method devised by Pichotka and Reichel.[9] The method consists of drawing blood from a vein by a 22 gauge needle connected to a capillary tube by a paraffin joint. The method is designed to limit the possible errors caused by surface and tissue factors. The capillaries were broken every 30 seconds. Three to 5 determinations were considered to be sufficient to give mean values under conditions of

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[*] The monosemicarbazone of adrenochrome.

[1] Cannon, W. B., and Mendenhall, W. L., *Am. J. Physiol.*, 1914, **34**, 225.

[2] Derouaux, G., and Roskam, J. Physiol. Soc., June 5, 1937, through *J. Physiol.*, *90*: Proc., 65, 1937.

[3] Braconier, F., LeBihan, H., and Beaudet, C., *Arch. Internat. de pharmacodyn. et therap.*, 1943, **69**, 181.

[4] Green, D. E., and Richter, D., *Biochem. J.*, 1937, **31**, 597.

[5] Derouaux, G., *Arch. Internat. de pharmacodyn. et therap.*, 1941, **66**, 202.

[6] Derouaux, G., *Arch. Internat. de pharmacodyn. et therap.*, 1943, **69**, 142.

[7] Bacq, Z. M., *Presse med.*, 1947, **16**, 175.

[8] Desfontaines, G., *Ann. biol. clin. Par.*, 1948, **6**, 138.

[9] Pichotka, J., and Reichel, H., unpublished data.

[†] Supplied by Squibb Institute for Medical Research, New Brunswick, N. J.

TABLE I.
Effect of Adrenoxyl on Clotting Time.

	Control							
Case	No. of determinations	Avg value (sec.)	Possible deviation (sec.)	Dose (mg)	Time after injection (min.)	No .of determinations	Avg value (sec.)	Possible deviation (sec.)
1	3	330	±30	.10	10-50	5	362	±52
2	3	470	±60	.45	10-45	4	420	±30
3	4	405	±45	.50	15-20	4	435	±75
4	3	370	±30	.45	15-20	4	367	±37
5	5	450	±60	.25	10-40	4	510	±45
Avg		405	±45				419	±45

TABLE II.
Effect of Adrenoxyl on Prothrombin Time.

Rabbit No.	Normal Prothrombin time (sec)	Dose	Post Adrenoxyl time (min.)	Prothrombin time (sec.)	Change
2	18 18 18 19 19	1	20	18 19	0
4	19 17 19 18 19	1.5	40	20 24 23 20	Increase, slight
8	17 17 17 17 16	3	45	clotted	- - - -
4	18 16 18 17	3	30	19 17 17 18 16	0
8	19 18 19 18 19	1	30	19 17 19 19 19	0
5	19 18	0.5	30	19 19	0

study. After the control measurements Adrenoxyl in a dosage of 0.10 to 0.50 mg was injected intravenously, and the clotting time of four to five separate blood samples was measured at intervals varying between 10 to 50 minutes later.

The clotting time had an average standard deviation of 4.4% in each subject in the control, and a mean standard deviation from the corresponding normal values of 4.8%. The individual differences between the pre- and post-Adrenoxyl findings fall within the range of physiological deviation.

2. *Prothrombin time* (Table II). Determinations were made on 5 rabbits before and after Adrenoxyl administration on blood samples obtained by intracardiac tap at stated intervals. The samples were collected in dry syringes and transferred immediately into test tubes containing 0.1 molar sodium oxalate. Prothrombin time was determined by the Quick method.[10] The applied dosage of Adrenoxyl varied between 0.0005 and 0.003 mg. No essential change was noted in the prothrombin time following Adrenoxyl injection.

3. *Pulse wave velocity* (Table III). Determinations were carried out by the photographic method of Frank[11] and Wexler.[12] Rubber membrane capsules and rubber tubes were used for air transmission of the impulses. The mirrors on the membranes were illuminated by a light beam from a projector, provided with a 3-slit lantern slide. The pulse curves of the carotid, femoral, and radial arteries were recorded by means of a photo-electric highspeed kymograph. The pulse wave velocity of the aorta (CA) and of the brachial (CB) artery was computed from the difference between the rise of the carotid, the femoral, and the radial curves. From these values the quotient, CB/CA, was calculated. Pulse rate and the blood pressure were also measured. Control determinations were followed by intravenous injections of 0.15 to 0.50 mg Adrenoxyl. The pulse rec-

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[10] Quick, A. J., *Amer. Journal Clin. Path.*, 1940, **10**, 222.

[11] Frank, O., *Z. f. Biol.*, 1903, **44**, 445; 1905, **46**, 441; 1929, **89**, 289.

[12] Wezler, K., *Z. f. Biol.*, 1935, **96**, 261; *Z. f. Breislaufforsch.*, 1935, **27**, 721.

16973

Effects of Adrenoxyl on Blood Coagulation Mechanism and Vasomotor Response.

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(Introduced by J. A. Shannon.)
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Cannon and Mendenhall[1] have shown that adrenalin shortens the coagulation time of blood. Roskam, Derouaux and associates[2] studied the effect of small doses of adrenalin on the bleeding time and reported a hemostatic action following the administration of the drug.

Adrenoxyl is a relatively stable derivative of adrenalin* developed by Braconier and associates.[3] Adrenochrome, adrenoxime,[4,5] and adrenoxyl,[6] oxidative derivatives of adrenalin, are reported as being capable of reducing the bleeding time approximately 25%. The adrenoxyl effect is demonstrable 15 minutes after intravenous injection in rabbits and 30 minutes after injection in man. The optimal doses are 0.001 mg in the rabbit and 0.1 to 0.5 mg in man. Derouaux[6] and Bacq[7] report that the circulation in rats and rabbits remains unaltered by the above doses of Adrenoxyl. These investigators emphasize lack of any sympathomimetic effect as reflected by the absence of change in the blood pressure and pulse rate in animals following injection of the drug. Roskam and associates believe that Adrenoxyl has a vasomotor effect which is responsible for the rapid occlusion of injured vessels. On the other hand, Desfontaines[8] has found a vasodilator effect of adrenochrome in man and recommends it for the treatment of hypertension.

As a preliminary study of the pharmacological properties of Adrenoxyl in relation to the bleeding time, we followed the administration of the drug with these screening procedures: Clotting time; prothrombin time; pulse wave velocity; skin temperature; and bleeding time determination. Such procedures would yield information concerning alterations in coagulability of blood and the vasomotor response of the injured vessels.

Methods and Results. Adrenoxyl prepared by Labaz Laboratories in ampules containing 0.1 and 0.5 mg in solution, was used in this investigation.† The prothrombin time and bleeding time determinations were performed on rabbits of both sexes. All other studies were made on healthy men between 17 and 39 years of age.

1. *Clotting time* (Table I). Blood samples were taken from the arm veins of 6 seated individuals and drawn directly into capillary tubes by a method devised by Pichotka and Reichel.[9] The method consists of drawing blood from a vein by a 22 gauge needle connected to a capillary tube by a paraffin joint. The method is designed to limit the possible errors caused by surface and tissue factors. The capillaries were broken every 30 seconds. Three to 5 determinations were considered to be sufficient to give mean values under conditions of

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* The monosemicarbazone of adrenochrome.

[1] Cannon, W. B., and Mendenhall, W. L., *Am. J. Physiol.*, 1914, **34**, 225.

[2] Derouaux, G., and Roskam, J. Physiol. Soc., June 5, 1937, through *J. Physiol.*, 90: Proc., 65, 1937.

[3] Braconier, F., LeBihan, H., and Beaudet, C., *Arch. Internat. de pharmacodyn. et therap.*, 1943, **69**, 181.

[4] Green, D. E., and Richter, D., *Biochem. J.*, 1937, **31**, 597.

[5] Derouaux, G., *Arch. Internat. de pharmacodyn. et therap.*, 1941, **66**, 202.

[6] Derouaux, G., *Arch. Internat. de pharmacodyn. et therap.*, 1943, **69**, 142.

[7] Bacq, Z. M., *Presse med.*, 1947, **16**, 175.

[8] Desfontaines, G., *Ann. biol. clin. Par.*, 1948, **6**, 138.

[9] Pichotka, J., and Reichel, H., unpublished data.

† Supplied by Squibb Institute for Medical Research, New Brunswick, N. J.

made over the ensuing 60 minute period. No significant reduction in bleeding time was noted following the injection of Adrenoxyl.

Discussion. Insofar as venous clotting time and prothrombin time reflect the speed of fibrin production, Adrenoxyl does not foster an accelerated process under the stated experimental conditions. It is, however, conceivable that tissue thromboplastic activity can be altered by Adrenoxyl and that the alteration is not evident where venous or heart sampling reduces the amount of tissue thromboplastin to the minimum.

After administration of Adrenoxyl, observations of the pulse rate, the blood pressure, the arterial wall tonus (as reflected by the pulse wave velocity), and the vaso-motor response of the superficial vessels (as evaluated by skin temperature determinations), do not reveal data which would suggest alteration in the vasomotor response that would not fall within normal physiological limits.

Bleeding time experiments are inconclusive since the experimental error of the technique used is of such great magnitude. We were unable to isolate real evidence that Adrenoxyl reduces the bleeding time under the stated experimental conditions. It is interesting to us to note that when an animal under light anesthesia responds to the stimulus of an incision on the ear, the bleeding time is reduced. This finding raises the question of advisability of collecting bleeding time data on unanesthetized animals.

Conclusions. 1. Under the stated experimental conditions Adrenoxyl does not influence (a) the speed of the coagulation process, and, (b) the vasomotor response.

2. The method of bleeding time determination in the experimental animal in our hands does not give conclusive results, therefore we are unable to confirm or deny the findings of Roskam and associates.

16974

Evaluation of Certain Dangers in the Use of Jet Injection Technic.

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As a substitute for the hypodermic syringe, a jet injector which utilizes the principles of the Diesel fuel injector has been described by Hingson and Hughes.[1] In the administration of substances by the hypodermic syringe, the possibility of an accidental intravenous injection is recognized. The present study was designed to investigate the possibility of a similar accidental intravenous injection with the "Hypospray."

Experimental Work. Dogs anesthetized with 30 mg per kg of sodium pentobarbital intravenously were given atropine sulfate by vein until electrical stimulation of the cut right vagus nerve failed to slow the heart. Both jugular veins were exposed by a medial approach so they could be seen by eversion of the edges of the incision. Blood pressure was continuously recorded by a mercury manometer connected to one carotid artery. Known doses of epinephrine were injected by syringe into the right jugular vein.

A "Hypospray"* with a 125 pound spring was loaded with a cartridge† containing 0.25 cc of a 0.4% solution of epinephrine hydrochloride. By eversion of the edge of the incision the left jugular vein was exposed to view. The tip of the cartridge was then pressed firmly against the skin on outside of the shaved neck and so aligned that the vein lay directly between the cartridge tip and the

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[1] Hingson, R. A., and Hughes, J. G., *Anesth. and Analg.*, 1947, 26, 221.

* Courtesy of the R. P. Scherer Corp., Detroit.

† Courtesy of the Gelatin Products Corp., Detroit.

TABLE III.
Effect of Adrenoxyl on Circulation.

Case	Dose	Time of determinations (min after inj.)	PR—Beats per minute	BP (mm Hg)	CA (m/sec.)*	CB (m/sec.)*	CB/CA
6	.15	Resting	74	120/70	6.37	7.45	1.17
		20	80	125/70	6.75	8.10	1.20
7	.20	Resting	60	100/65	4.05	4.95	1.22
		10	60	115/70	4.12	5.03	1.22
8	.20	Resting	81	120/75	5.55	6.42	1.16
		10	70	120/70	5.18	5.77	1.11
9	.10	Resting	69	110/70	5.90	7.97	1.35
		60	67	110/70	5.74	7.80	1.36
10	.10	Resting	51	115/70	6.27	6.41	1.02
		50	55	115/70	6.25	6.49	1.04
11	.50	Resting	52	110/70	6.63	9.54	1.44
		15	54	110/70	6.26	9.14	1.46
12	.50	Resting	45	110/70	5.50	7.62	1.38
		15	43	115/70	5.78	7.68	1.33
13	.50	Resting	66	120/75	6.80	9.04	1.31
		15	67	120/75	7.04	9.12	1.30

* Meters per second.
PR—Pulse rate.
BP—Blood pressure.
CA—Pulse wave velocity in aorta.
CB—Pulse wave velocity in brachial artery.

TABLE IV.
Effect of Adrenoxyl on Skin Temperature.

Case	Dose (mg)	Time after injection (min.)	Mean skin temp. (°C) Normal	Mean skin temp. (°C) Adrenoxyl	Room temp. (°C)
12	.5	40	31.85	31.44	28.7
14	.5	25	31.11	31.01	26.9
15	.5	30	31.60	31.73	27.7
16	.5	40	30.80	30.70	27.9

ords were repeated immediately and 10-50 minutes later. During the whole procedure the subjects were maintained in the supine position. Following the injection of Adrenoxyl, deviations of the pulse wave velocity were negligible. Maximal change of the quotient, CB/CA, did not exceed 35%, which falls within physiological variations.

4. *Skin temperatures* (Table IV). Readings were taken from undressed recumbent subjects by means of a Dermalor instrument having a standard deviation of ± 0.2° C. The control readings were performed 30, 40, and 50 minutes after the undressing, in a room temperature of 27° to 29° C. The skin temperature measurements were repeated 20, 30, and 40 minutes after an intravenous injection of 0.5 mg Adrenoxyl. The mean value was determined for each selected body site (forehead, ears, nose, chin, shoulders, forearm, hand, fingers, umbilicus, knee, mid-leg, feet, and toes) on the basis of 3 "control" and 3 "test" measurements. Skin temperature determinations remained essentially unchanged following administration of Adrenoxyl.

5. *Bleeding Time.* Determinations were made on 12 rabbits anesthetized by sodium amytal, intravenously administered. Small, uniform, through and through incisions were made along the caudal margin and tip of the ears. The incised wounds were placed under a uniform saline drip. The time was recorded between the moment of incision and the moment of cessation of bleeding. The error of cutting a large vessel was avoided by transillumination. After a satisfactory control time was established, Adrenoxyl (0.001 to 0.0025 mg) was injected intravenously and periodic bleeding time determinations were

Conversely, with direct hits the vein was seen to balloon out whether it was originally full or empty. Firm pressure of the cartridge tip against the tissues did not prevent such ballooning. After having passed through the proximal vein wall, the jet had lost so much velocity that much of it failed to penetrate the distal vein wall and thus remained in the lumen.

Inspection of Table I shows that the amount of the drug retained was not appreciably influenced by whether or not the vein was empty or full. In larger animals the veins are larger and are easier to hit through the diameter. Also the thicker vein walls decreased the jet velocity and less escaped through the distal wall. Since man has numerous large superficial veins, his risk of an intravenous injection may be greater than that of the dog when the jet is used.

The portion of a vein penetrated by a jet of epinephrine became blanched and constricted for several minutes. Sometimes small clots appeared on the vessel at the points of entrance and exit of the jet.

The shape of the blood pressure curve resulting from a jet injection of epinephrine differed from that following needle injection. With direct hits, the ascending limbs of the curves were similar, but the blood pressure always returned to the control level more slowly after a jet injection. This is taken to indicate: (1) a gradual entrance into the lumen of epinephrine lodged in the wall of the vein, (2) entrance of additional small quantities lodged in the small tributaries, (3) rapid absorption of epinephrine trapped in adjacent tissues (*cf.* Clark.[9]).

Massage of a jet injected muscle, connective tissue or even the vein itself elevated the blood pressure. In one case, the blood pressure rise induced by massage of a jet injected area was 12 mm of Hg, but lower responses were usually obtained. Luckhardt and Koppanyi[10] have reported a similar elevation of blood pressure from massage of areas containing needle-injected epinephrine.

As the blood pressure slowly returned to the control level, cardiac irregularities, showing as dropped beats, were much more frequent from jet injections than from needle injections. It is not known whether the dropped beats indicated premature systoles of the ventricles or heart block.

Ten attempts to make blind intravenous injections with the "Hypospray" at the junction of the jugular and subclavian veins or into the femoral vein were not successful.

Conclusions. 1. Under conditions very favorable for intravenous injection, the "Hypospray" has lodged as much as 97% of the total available drug in the vein, even when administered through the skin and subcutaneous tissues.

2. Large veins are easier to hit than small veins and retain a larger amount of the jet-injected material.

3. The probability of unintentionally penetrating a major vessel with the jet is small but the risk is always present.

4. Jet discharges should not be made in the neighborhood of large vessels unless the drug to be injected is safe for intravenous administration.

5. The jet may produce macroscopically visible damage to a vessel.

The author wishes to thank Drs. J. P. Quigley and R. A. Woodbury for their interest and help.

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[9] Clark, A. J., and Raventós, J., *Quart. J. Exp. Physiol.*, 1939, **29**, 185.

[10] Luckhardt, A. B., and Koppanyi, T., PROC. SOC. EXP. BIOL. AND MED., 1926, **23**, 774.

TABLE I.
Summary of Jet Injections Lodging as Much as One Per Cent Intravenously.

		Blood pressure (mm Hg)		μg epinephrine base lodged in vein			
Dog No.	Wt	Control	Maximum	Per kg	Per dog	%*	State of vein
1	5.5	96	226	18.74	103	15.2	D†
2	15.9	126	220	.49	8	0.9	D
		144	315	51.48	818	97.7	Empty
		135	306	35.32	562	66.8	D
3	5.0	128	247	10.34	52	6.2–	Empty
		122	240	8.09	40	4.8	D
4	4.7	108	188	3.38	16	1.9	D
		110	195	3.48	16	2.0	D
5	16.0	153	206	.52	8	1.0–	D

* Per cent of the total epinephrine available, *i.e.*, 833 μg.
† Distended.

incision. Any of the jet passing through the vein would escape into the room or be sponged out at once. The instrument was then discharged. The vein rested directly on the subcutaneous tissue or else was separated from it by about 5 mm of muscle. For some jet injections the jugular vein was emptied; for others the vein was distended by clamping and the clamps were removed immediately after the discharge.

A dose-effect curve was fitted for each animal by the method of least squares, using the equation: maximum blood pressure attained is equal to a constant multiplied by the dose per kilogram raised to a constant power. Accurate assays of epinephrine can be made from such dose-effect curves.[2-8] The maximum blood pressure reached after each jet discharge of epinephrine was measured. From the dose-effect curve or its equation the quantity of epinephrine required to produce the given maximum pressure can be determined either graphically or by calculation. The results of 12 jet injections which lodged intravenously as much as 1% of the epinephrine in the cartridge are listed in Table I; 22 discharges which lodged less than 1% are omitted.

Results and Discussion. The greatest amounts of the drug which lodged in the vein in 34 jet injections were 97.7, 66.8, 15.2, and 6.2% of the total discharged. Of these, the first and fourth quantities lodged in empty veins; the second and third lodged in distended veins. Thus if the jet is discharged over a large vein a significant fraction of the discharged drug may be administered intravenously.

When the "Hypospray" is used, several factors appear to determine the fraction of the epinephrine which lodges in the veins: (1) alignment of the blood vessel with the jet, (2) amount of pressure applied to the tissues, (3) diameter of the vein, (4) amount and toughness of the tissues separating the nozzle and the vein lumen, (5) ability of the vein to roll away from the discharge under impact of the jet.

Even though the vein was visible, we were not always successful in discharging the jet through the diameter of the vein. When the jet nicked the vein edge, little of the drug entered the lumen as the major portion was deflected. Further, such off-center discharges tended to roll the vein to one side.

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[2] Elliott, T. R., *J. Physiol.*, 1904-5, **32**, 401.

[3] *Ibid.*, 1912, **44**, 374.

[4] Lyon, D. M., *J. Pharmacol. and Exp. Therap.*, 1923, **21**, 229.

[5] Rosenblueth, A., *Am. J. Physiol.*, 1932, **101**, 149.

[6] Clark, A. J., Mode of Action of Drugs on Cells, Baltimore, 1933.

[7] Hjort, A. M., de Beer, E. J., and Randall, L. O., *J. Pharm. and Exp. Therap.*, 1941, **71**, 105.

[8] Brown, R. V., *Fed. Proc.*, 1948, **7**, 208.

TABLE I.

Incidence of Breast Tumors and the Time of Appearance of the Neoplasms in the Various Experimental Groups.

Group	Castrates bearing	Age at transplantation	No. of mice	Age at death of all mice (mo.)		Animals with breast cancer			
						No. of cases	%	Age (mo.) of appearance	
				Mean	Range			Mean	Range
I.	Hypophyseal transplants	1 mo.	28	16.1	8-21	0	0	—	—
II.	Ovarian transplants	1 "	27*	16.9	6½-22	1	5	6.5	—
III.	Hypophyseal and ovarian transplants	1 "	26	17.1	8-22	8	32.5	12.9	8-20
IV.	Hypophyseal transplants	7 "	28	19.1	8-25	0	0	—	—
V.	Ovarian transplants	7 "	26†	15.9	7½-20	0	0	—	—
VI.	Hypophyseal and ovarian transplants	7 "	29	15.8	9-24	0	0	—	—

*7 animals still alive without tumor } 21-24 mo. old
†4 " " " " "

The wounds were subsequently closed with silk sutures and the animals caged in groups of 4.

The mice were fed a stock diet of Purina Laboratory Chow and water available at all times; they were examined at regular intervals for the appearance of breast tumors or evidence of leukemia. Animals that had developed tumors or were suspected of having leukemia or any other disease were killed. At necropsy, special care was taken to recover the transplants, and definite grafts, as well as tissues suggestive of being remnants of grafted tissue were preserved for microscopic examination. In addition, mammary glands, pieces of liver, spleen, kidneys, lung, heart, thyroid, adrenals, hypophysis, prostate and some bones were removed as well as any pathological structures that were found. The histological findings and the data on leukemia will be reported elsewhere.

Observations. The results of our experiments are presented in Table I.

Age group A. Animals castrated at the age of 3 to 4 weeks and receiving grafts at the age of one month:

I. *Castrates bearing hypophyseal transplants* (28 animals) died or were killed at a mean age of 16.1 months with a range from 8 to 21 months. None of these animals showed a breast cancer. II. *Castrates bearing ovarian transplants* (27 animals) reached a mean age of 16.9 months and a range from 6½ to 22 months. One of these animals (5%) developed a breast cancer at 6½ months of age. At the time of this report 7 animals of this group, 22 to 24 months old, are still alive and without tumor. III. *Castrates bearing ovarian and hypophyseal transplants* (26 animals): The mean age at death was 17.1 months with a range from 8 to 22 months. In 8 mice (32.5%), breast cancers were found. The mean age at death of tumor-bearing animals was 12.9 months, the earliest tumor appearing at 8, the latest at 20 months of age.

Age Group B. Animals castrated at the age of 3 to 4 weeks and receiving grafts at the age of 7 months:

IV. *Castrates bearing hypophyseal transplants* (28 animals) reached a mean age of 19.1 months with a range from 8 to 25

16975

Mammary Cancer in Castrate Male Mice Receiving Ovarian and Hypophyseal Grafts at Various Ages.*

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In young virgin mice of susceptible strains, grafts of anterior hypophysis stimulate the growth of the mammary gland and increase the incidence of breast cancer.[1] Anterior hypophyses grafted together with ovaries are ineffective in young males with intact testicles; however, these transplants induce mammary cancer in young castrate males. This carcinogenic effect has been attributed to the gonadotropin given off by the transplanted hypophyses and stimulating the output of estrogenic hormone by the animal's own or by the grafted ovaries.[1,2] This view is based on the fact that in the absence of ovaries hypophyseal grafts are ineffective. The reaction of the breast tissue of male castrates to transplanted ovaries alone is definite, and the number of mammary tumors appearing in these animals may even exceed the number of spontaneous breast cancers found in virgin females of the same strain.[2-5] Moreover, the response of the male breast to injections of estrogenic hormone depends upon the age of the animal at the beginning of the treatment. A maximum susceptibility was noted if the treatment was started about the onset of sexual maturity. Loeb[6] attributed this age effect to the more marked growth reactivity of the breast of younger animals and possibly—since in females such an age factor could not be demonstrated—to an inhibiting action exerted by the male sex hormone. We found this age effect to be due only partly to the action of the testicle, since in castrates of various ages it was also present, though to a lesser degree.[7]

The present experiments were carried out in order to determine whether or not an age factor operates also in the production of breast cancer in male castrates bearing grafts of ovaries alone or in combination with anterior hypophyses.

Material and Methods. One hundred sixty-four male mice of the closely inbred strain A raised in our laboratory and originating in the Bar Harbor colony were used. The animals were castrated at 3 to 4 weeks of age and received the following grafts at the age of 1 or 7 months respectively: (a) 4 anterior hypophyses (27 young and 28 adult animals); (b) 2 or 4 ovaries (28 young and 26 adult animals); (c) 4 anterior hypophyses and 2 or 4 ovaries (26 young and 29 adult animals). The organs to be grafted were usually obtained from mice 2 to 3 months old and closely related to the host, that is, from brothers, sisters, and cousins (syngenesiotransplants)[1]; in 38 instances, non-related donors (homoiotransplants) were used. At the time of transplantation, the hypophyses and ovaries were removed from the donor animals under sterile precautions and kept in sterilized Ringer's solution for a few minutes until two subcutaneous pockets had been prepared on either side of the chest wall of the hosts. The grafts were then transferred into these pockets, half of them to the right and the other half to the left side.

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* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

[1] Loeb, L., and Kirtz, M. M., *Am. J. Cancer*, 1939, **36**, 56.

[2] Loeb, L., Blumenthal, H. T., and Kirtz, M. M., *Science*, 1944, **99**, 230.

[3] Murray, W. S., *J. Canc. Res.*, 1928, **12**, 18.

[4] de Jongh, S. E., and Korteweg, R., *Acta brev. Neerl.*, 1935, **5**, 12.

[5] Huseby, R. A., and Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 32.

[6] Loeb, L., *Biol. Sympos.*, 1945, **11**, 197.

[7] Silberberg, M., and Silberberg, R., (a) PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 438; (b) *Arch. Pathol.*, in press.

if anterior hypophyses were grafted together with ovaries. Transplantation of anterior hypophyses alone failed to produce breast cancer. No mammary tumors occurred in castrates into which ovaries, anterior hypophyses alone or anterior hypophyses in combination with ovaries were grafted at 7 months of age. Thus in male mice, an age factor which is independent of the testicle operates in the production of breast cancer by ovarian and anterior hypophyseal grafts.

16976

Effects of Restricted Feed Intake in Intact and Ovariectomized Rats on Pituitary Lactogen and Gonadotrophin.[*]

Joseph Meites and Jack Owen Reed. (Introduced by F. W. Bernhart.)

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Starvation generally has been shown to result in decreases in the weights and functions of the endocrine glands, and these decreases have been attributed to a reduction in secretory activity by the anterior pituitary.[1-6] The adrenals represent an exception, since in cases of extreme or total starvation their weight and activity are actually augmented rather than reduced.[1,7,8]

Assays of the gonadotrophin content of the pituitaries of starved rats have revealed a decrease in the hands of some workers,[4,5] while others have reported no change in pituitary content.[9,10] The lactogenic hormone content of the pituitary was found to be reduced during complete starvation in lactating rats.[11]

It was the purpose of these experiments to seek further clarification of the effects of restricted food intake on the pituitary content of these two hormones, since under other conditions the latter has served as a reliable index of the amounts of these hormones present in the blood stream.[10-13] It was also considered desirable to determine the above effects in both intact and ovariectomized rats, since in the latter the pituitary gonadotrophin content is known to be higher[14-16] and pituitary lactogen lower than in intact rats.[13,17]

Methods. Forty-eight intact female rats of about 200 g body weight and 35 rats which had been ovariectomized about a month previous and weighed about 230 g were used in months. None of these mice had breast cancer. V. *Castrates bearing ovarian transplants* (26 animals) did not do well, the mean age at death being 15.9 months with a range from 7½ to 20 months. Again, no breast cancer appeared in any of the animals. Four mice of this group 21 months old and older are still alive and without tumor at the time of writing. VI. *Castrates bearing ovarian and hypophyseal transplants* (29 animals) reached a mean age of 15.8 months with a range from 9 to 24 months. None of these mice had a mammary cancer.

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[*] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 1023 (N.S.).

[1] Mulinos, M. G., and Pomerantz, L., *J. Nutrition*, 1940, **19**, 493.

[2] Mulinos, M. G., and Pomerantz, L., *Endocrinology*, 1941, **29**, 558.

[3] Mulinos, M. G., and Pomerantz, L., *Endocrinology*, 1941, **29**, 267.

[4] Mason, K. E., and Wolfe, J. M., *Anat. Rec.*, 1930, **45**, 232.

[5] Werner, S. C., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 101.

[6] Stephens, D. J., and Allen, W. M., *Endocrinology*, 1941, **28**, 580.

[7] Mulinos, M. G., and Pomerantz, L., *Am. J. Physiol.*, 1941, **132**, 368.

[8] Boutwell, R. K., Brush, M. K., and Rusch, H. P., *Am. J. Physiol.*, 1948, **154**, 517.

[9] Marrian, G. F., and Parkes, A. S., *Proc. Roy. Soc. London*, 1929, **105b**, 248.

[10] Maddock, W. O., and Heller, C. G., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 595.

[11] Meites, J., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 416, 1948.

[12] Meites, J., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 190.

[13] Meites, J., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 415, 1948.

[14] Engle, E. T., *Am. J. Physiol.*, 1929, **88**, 101.

[15] Evans, H. M., and Simpson, M. E., *Am. J. Physiol.*, 1929, **89**, 371.

[16] Wolfe, J. M., *Am. J. Anat.*, 1932, **50**, 351.

[17] Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 266, 1937.

Tumor development was independent of the number of ovaries grafted inasmuch as tumors were found in animals that had received 2 or 4 ovaries in addition to the hypophyseal transplants. The presence or absence of tumor could not be correlated to the state of preservation of the grafted tissue at the time of necropsy. In a number of animals without breast cancer functioning transplants were found at the time of necropsy, whereas in some animals with breast cancer no remnants of transplanted tissue could be detected. The degree of relationship of host and donor apparently did not influence the development of tumors, since breast cancer was found in animals bearing grafts from unrelated donors. This is probably due to the fact that the strain had been closely inbred for years and that the individuals have reached a state of close genetic similarity.

Discussion. The incidence of spontaneous breast cancers in breeding females of our strain A stock was 49%. The earliest breast cancer appeared at 6½ and the latest at 20 months of age with a mean age of 11.4 months. No spontaneous breast cancers have developed in our virgin females of strain A.

No breast cancer was observed in castrate male mice of strain A bearing transplants of anterior hypophyses. This indicates that no direct stimulation was exerted on the mammary tissue by hypophyseal activity or that any existing stimulus[8,9] was too weak to induce cancerous growth.

Of 20 male castrates bearing ovarian grafts one developed a breast cancer. This incidence is considerably lower than that observed by Loeb[2] and by Huseby and Bittner,[5] but approaches more closely the results of Murray.[3] The comparatively small number of carcinomas observed in our experiments is also in agreement with the failure of our virgin female mice of this strain to develop spontaneous mammary cancer. Moreover, castrate male mice showed a maximum susceptibility to estrogen-induced cancer if the injections were begun about the age at which sexual maturity is reached in animals with intact testicles.[7] Similar conditions may exist in regard to estrogenic hormone secreted by the transplanted ovaries. The mice used in the present investigation might not have reached the age of maximum susceptibility at the time the transplants began to function. The susceptibility of the breast tissue at the onset of stimulation seems to play a more important role even than the length of time during which the stimulus is allowed to act. The only tumor found in castrate males bearing ovarian transplants occurred at the age of 6½ months, that is 5½ months after grafting and as early as the earliest cancer in our breeding females. Apparently, the breast tissue of this animal was, for reasons unknown, so highly sensitized that a comparatively slight stimulus of relatively short duration sufficed to produce cancerous growth. In castrate male mice bearing hypophyseal transplants in addition to ovarian grafts the breast cancer rate was 32.5%, that is, about 6½ times as high as in castrates bearing ovarian transplants only. This indicates a marked stimulation of ovarian activity by the grafted anterior hypophyses.

No mammary tumors were found in castrate male mice receiving endocrine transplants at the age of 7 months. This confirms our earlier observations that there is with advancing age a loss of sensitivity of the mammary tissue to hormonal stimulation independent of the testicle.[6,7]

Summary. Syngenesiotransplants of ovaries made at one month of age caused mammary cancer in 5% of castrate male mice of strain A. The tumor incidence rose to 32.5%,

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[8] Gomez, E. T., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 607.

[9] Mixner, J. P., and Turner, C. W., *Endocrinology*, 1942, **30**, 591.

TABLE II. *Assays of the Gonadotrophin and Lactogen Content of Pituitaries of Intact and Ovariectomized Rats on Restricted Feed Intakes.*

Intact Donor Rats Procedure	Avg pit. wt, mg	Body wt, g	Recipient rats* (gonadotrophin assays) Ovarian wt per rat	Ovarian wt per 100 g body wt	Uterine wt per rat	Uterine wt per 100 g body wt	Recipient pigeons‡ (lactogenic assays) Avg Reece-Turner units per pit.	units per mg pit.
Controls, fed *ad lib.*	15.2	77	18.6 ± 2.3†	24.2	53.3 ± 2.5†	69.5	4.5	0.30
Fed ¾ *ad lib.*	11.7	77	19.2 ± 2.5	25.0	71.9 ± 12.0	93.8	4.0	0.34
,, ½ ,, ,,	10.9	75	17.8 ± 2.2	23.8	72.4 ± 9.2	87.0	3.0	0.28
,, ¼ ,, ,,	9.1	78	20.0 ± 2.3	22.7	88.6 ± 3.9	92.5	2.5	0.27
No feed	8.9	81	17.8 ± 2.4	24.8	65.0 ± 9.1	110.0	2.2	0.25
Controls uninjected		74	15.2 ± 1.8	20.5	23.4 ± 4.6	31.0		
Ovariectomized Donor Rats								
Controls, fed *ad lib.*	11.3	68	65.7 ± 6.2	96.3	84.4 ± 5.0	123.8	3.1	0.27
Fed ¾ *ad lib.*	13.2	74	63.5 ± 11.8	85.8	116.0 ± 7.0	158.0	3.1	0.23
,, ½ ,, ,,	11.8	71	59.6 ± 7.2	84.1	85.0 ± 10.0	120.0	2.5	0.21
,, ¼ ,, ,,	11.1	73	65.6 ± 5.5	90.1	99.4 ± 5.4	136.5	1.8	0.16
No feed	11.7	72	65.7 ± 6.2	96.3	84.4 ± 5.0	123.8	1.8	0.15

* The equivalent of one donor rat pituitary was injected into each recipient rat.

‡ The equivalent of two donor rat pituitaries was injected into 5 pigeons.

† Standard error of the mean $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

On a 100 g body weight basis, pituitary weight was increased in all restricted diet groups; thyroid weight was slightly reduced on the ¾ and ½ feed regimes but increased on the ¼ and no-feed regimes; and adrenal weight was unchanged on ¾ and ½ feed intake and increased on ¼ and no-feed intake.

These differential effects of restricted food intake on endocrine weights in intact and ovariectomized rats are interesting since they have not been reported previously. This may be due to the fact that almost all of the previous work on this problem has been done in intact animals. It is not known whether such differential effects would be obtained in intact and ovariectomized rats during more prolonged periods of feed restriction.

The effects of reduced feed intake on the lactogenic and gonadotrophic hormone content of the pituitaries are given in Table II. In neither the intact nor ovariectomized rats on restricted feed allowances was the gonadotrophin content of the pituitaries reduced from that of the controls fed *ad libitum*. In fact the concentration was actually increased on the basis of hormone content per milligram of pituitary in the intact groups (since pituitary weight was decreased on the restricted diets). The pituitaries of the ovariectomized rats contained about 10 times as much gonadotrophin as the intact rats, as indicated by their ability to increase ovarian weight in the recipient immature rats.

The lactogenic potency of the pituitaries of both intact and ovariectomized rats on reduced feed was decreased below that of the controls fed *ad libitum*, on an individual basis. The decrease in lactogenic hormone was roughly proportional to the decrease in feed intake. On the basis of hormone concentration per milligram of pituitary there was only a slight decrease in the intact groups and a marked decrease in the ovariectomized groups. In corroboration of previous findings[13,17] the lactogen content of the pituitaries of the ovariectomized control rats was reduced below that of the intact controls.

Discussion. Maddock and Heller[10] have suggested a possible cause for the divergent results reported by different workers on the

TABLE I. Weights of Endocrine Glands of Intact and Ovariectomized Rats on Restricted Feed Intakes.

No. rats per gp.	Procedure	Orig. body wt, g	Final body wt, g	% diff. in body wt	Ovarian wt per rat, mg	Ovarian wt per 100 g body wt, mg	Uterine wt per rat, mg	Uterine wt per 100 g body wt, mg	Adrenal wt per rat, mg	Adrenal wt per 100 g body wt, mg	Thyroid wt per rat, mg	Thyroid wt per 100 g body wt, mg	Pituitary wt per rat, mg	Pituitary wt per 100 g body wt, mg
	Intact Rats.													
10	Controls fed *ad lib.*	196	220	+12	62.4	28.4	393.4	179.0	63.2 ± 1.8*	28.8	18.1 ± 0.9*	8.2	15.2 ± 1.2*	6.9
10	Fed ¾ *ad lib.*	193	197	+ 2	52.3	26.6	374.5	190.4	54.0 ± 2.5	27.5	15.9 ± 0.9	8.1	11.7 ± 0.6	6.0
10	,, ½ ,, ,,	197	173	—12	50.5	29.1	256.0	147.7	47.3 ± 2.0	27.3	12.6 ± 0.6	7.3	10.9 ± 0.4	6.3
9	,, ¼ ,, ,,	200	153	—24	46.7	30.5	154.3	100.9	47.7 ± 3.0	31.2	13.0 ± 0.5	8.5	9.1 ± 0.2	6.0
9	No food	209	141	—33	51.1	36.2	184.0	130.5	61.8 ± 3.6	43.8	10.6 ± 0.2	7.5	8.9 ± 0.4	6.3
	Ovariectomized Rats.													
7	Controls fed *ad lib.*	230	248	+ 8					57.8 ± 3.0	23.1	21.9 ± 1.6	8.8	11.3 ± 0.8	4.6
7	Fed ¾ *ad lib.*	230	221	— 4					51.3 ± 1.9	23.2	16.1 ± 1.2	7.3	13.2 ± 0.7	5.9
7	,, ½ ,, ,,	228	194	—15					48.0 ± 3.3	24.7	15.8 ± 1.7	8.1	11.8 ± 0.5	6.1
7	,, ¼ ,, ,,	226	160	—30					52.3 ± 2.4	32.7	15.0 ± 1.1	9.4	11.1 ± 0.6	7.0
7	No food	234	162	—30					60.9 ± 1.6	37.6	16.8 ± 1.4	10.4	11.7 ± 0.5	7.2

* Standard error of the mean $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

these experiments. All were of the Sherman strain. The intact and ovariectomized rats were divided into 5 groups each, and were put on *ad libitum*, ¾, ½, ¼ and no-feed regimes. The ¾, ½ and ¼ feed levels were determined daily from the *ad libitum* intake of the control group. The *ad libitum* and partially-fed groups were killed at the end of 14 days and the unfed group at the end of 7 days. The endocrine glands were removed, trimmed free of extraneous tissue, and weighed on a Roller-Smith balance.

The pituitaries from each group were macerated into a fine paste with a small mortar and pestle. Distilled water was then added to a volume of one ml per pituitary, and the fine suspension was taken up with a syringe through a number 25 needle.

Gonadotrophic potency was measured by injecting the equivalent of 5 pituitaries from each group into 5 immature female rats, making two injections daily for 5 days. On the 6th day the rats were killed and the ovaries and uteri were weighed. Lactogenic potency was assayed by injecting the equivalent of 2 pituitaries from each group intradermally over the crop glands of 5 mature White Carneau pigeons during a 4-day period, and rating the crop responses by the Reece-Turner[17] method on the 5th day.

Results. Although incidental to the main problem, the effects of restricted feed intake on the weights of the endocrine glands will be considered first (*Table I*). In the intact rats, the weights of the pituitary, thyroid, adrenals and ovaries were reduced on an individual basis in all the restricted diet groups, with the exception of the adrenal weights of the completely starved group. On a 100 g body weight basis, the weights of these glands remained the same, again with the exception of the adrenal weights of the completely starved group. These results agree on the whole with those previously reported by other workers.

In the ovariectomized rats, there was no loss of pituitary weight in any of the restricted diet groups; a slight but similar loss of thyroid weight in all restricted diet groups; and a slight loss of adrenal weight in all restricted diet groups except the non-fed group.

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Discussion. Maddock and Heller[10] have suggested a possible cause for the divergent results reported by different workers on the

assay of pituitary gonadotrophin content in starved rats. They pointed out that other investigators implanted entire pituitary glands into recipient assay rats, whereas they injected suspensions of pituitary tissue, as was also done in our experiments. The implantation method is believed to be less exact since necrosis, growth, or encapsulation may occur, resulting in an exceedingly variable delivery of gonadotrophic hormone.

It is not clear as to why the pituitary gonadotrophin content remains the same during periods of starvation while pituitary lactogen becomes reduced. There can be no doubt that starvation, depending on its degree, results either in a reduction or complete cessation of gonadal and mammary gland activity. This would certainly seem to indicate that the amounts of circulating gonadotrophic and lactogenic hormones are reduced.

On the assumption that pituitary content of a hormone represents the resultant between the amount being produced and the amount released into the blood stream, Maddock and Heller[10] explain the 'dichotomy' between gonadotrophin content in the pituitary and blood stream of starved rats as being due to a failure of release and to a minimum of production by the pituitary. The decrease of lactogenic hormone content in the pituitary during starvation could be explained as being due primarily to a failure of production with little or no interference with the release mechanism.

Assays of pituitary content of gonadotrophin have proven to be reliable indicators of the amounts present in the circulation following ovariectomy, during estrogen administration, but not during starvation. Assays of the lactogen content of the pituitary have accurately reflected the amounts present in the blood stream during lactation, during estrogen or testosterone administration, and also during periods of restricted feed intake.

Summary. The lactogenic and gonadotrophic hormone content of pituitaries of intact and ovariectomized rats was determined after full, ¾, ½, ¼ and no-feed regimes. The full or partially-fed groups were sacrificed at the end of 14 days and the unfed group at the end of 7 days. Pituitary lactogen content was reduced in the intact and ovariectomized rats on the ½, ¼ and no-feed regimes below that of the ¾ and full-fed controls. No change occurred in the gonadotrophin content of the pituitaries of either the intact or ovariectomized rats, regardless of the level of feed intake. In the intact but not in the ovariectomized rats, restricted feed intake caused a marked reduction in the weights of the pituitary, thyroid and adrenals, except that on the no-feed regime adrenal weight was increased in both groups.

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Assay of Secretin and Cholecystokinin Concentrates.*

LEON L. GERSHBEIN, C. C. WANG, AND A. C. IVY.

From the Department of Clinical Science, University of Illinois College of Medicine, Chicago

The most feasible assay of secretin concentrates involves the cannulation of the pancreatic duct and observation of the increased rate of pancreatic juice output upon intravenous injection of the sample. Ivy, Kloster, Drewyer and Lueth[1] introduced the threshold dose, namely, that amount of a secretin concentrate causing a flow of 10 drops (0.4 ml) of pancreatic juice within a 10-minute period over the control rate in the dog. In a later work, Greengard and Ivy[2] reported that the threshold dose of a preparation obtained from hog duodenums (SI) and adopted as a standard was 0.25 mg for many dogs. This

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* This study has been aided in part by a grant-in-aid from the National Institute of Health, Bethesda, Md.

[1] Ivy, A. C., Kloster, G., Drewyer, G. E., and Lueth, H. C., *Am. J. Physiol.*, 1930, **95**, 37.

[2] Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 427.

value was employed for assay in conjunction with the ratio of weights of unknown to standard eliciting a 10 drop response in 10 minutes. Still[3] determined secretin unitage from the ratio of drops of unknown to a vasodilatin-free standard produced by the injection of 4 mg of each. Neither sample data nor comments as to reproducibility were given. Penau and Simonnet[4] derived a unit from uniform injections of secretin concentrates and measurement of juice output after 15, 30 and 45 minutes. A high variability resulted. A marked departure from the above direct methods is that of Wilander and Ågren[5] who employed narcotized cats. A unit was arrived at from the titration of the alkalinity of secretions from the duodenum soaked up by a rolled-up filter paper. Among others, a criticism of this method is that the alkalinity is not due to pancreatic juice alone. A comparison of this cat unit with the one obtained by Greengard and Ivy has been made.[2,6]

A direct method for the assay of cholecystokinin (CCK) fractions described by Ivy and Oldberg[7] involves cannulation of the gall bladder after clamping the cystic duct. Contractions were observed by connection to a recording tambour. A unit of CCK potency was defined as that amount of vasodilatin-free concentrate which when injected intravenously brings about a gall bladder contraction of one centimeter of bile. Recently[8] a method has been reported for CCK estimation which involves the recording of an increase in the pressure in the common bile duct after the injection of CCK in unanesthetized dogs, the common bile duct being cannulated through a duodenal fistula. The *in vitro* contraction of guinea pig[9] and frog[10] gall bladders has been used for assaying CCK preparations. However, numerous difficulties are encountered with this method, notably, the regulation of intravesical pressures. In the present report, details are given for the assay of secretin and CCK, both of which may be carried out on the same dog. New reproducible units of potency are derived for these hormones by comparison of the gall bladder or pancreatic response produced by the standard with that of a test concentrate.

Preparation of the Standard Concentrate. A representative batch of SI prepared by treatment of a hog duodenal extract with trichloroacetic acid according to the method of Greengard and Ivy[2] was adopted as the standard. Although a few products proved to be somewhat more potent, the standard concentrate was chosen on the basis of the absence of vasodilatins. The dry standard has been stored at room temperature for over 2 years with no detectable loss in potency. A freshly prepared aqueous or dilute hydrochloric acid solution is used in the assay.

Assay Procedure. Dogs, 9-13 kg in weight, which have been fasted for at least 19 hours are placed under pentobarbital anesthesia. The main pancreatic duct is cannulated and after filling of the cannula, a rubber or plastic tube containing saline is connected so as to register juice output on a drop recorder. Carotid blood pressure is recorded throughout. A priming dose of secretin concentrate is injected into the exposed femoral vein, and, after some time, the basal rate of flow is determined. This is useful in the exploration of a practical drop rate especially where a pancreas is hypersecretive. A known amount of standard SI is injected and the number of drops of pancreatic juice, which decreases rapidly in rate with time, is recorded over a 10-minute period. After basal conditions prevail, the test concentrate is then administered in an amount yielding the same drop count within one to two drops over the same time interval. The ratio of standard to test sample dosages is assay of pituitary gonadotrophin content in starved rats. They pointed out that other investigators implanted entire pituitary glands into recipient assay rats, whereas they injected suspensions of pituitary tissue, as was also done in our experiments. The implantation method is believed to be less exact since necrosis, growth, or encapsulation may occur, resulting in an exceedingly variable delivery of gonadotrophic hormone.

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[3] Still, E. U., *Physiol. Rev.*, 1931, **11**, 342.

[4] Penau, H., and Simonnet, H., *Cong. Pharm.* (Liège, 1934), **1935**, 145; *C. A.*, 1936, **30**, 4893.

[5] Wilander, O., and Agren, G., *Biochem. Z.*, 1932, **250**, 489.

[6] Greengard, H., and Stein, I. F., Jr., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 149.

[7] Ivy, A. C., and Oldberg, E., *Am. J. Physiol.*, 1928, **86**, 599.

[8] Snape, W. J., Friedman, M. H. F., and Thomas, J. E., *Gastroenterology*, 1948, **10**, 496.

[9] Jung, F. T., and Greengard, H., *Am. J. Physiol.*, 1933, **103**, 275; Doubilet, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 379; Agren, G., *Skand. Arch. Physiol.*, 1934, **81**, 234.

[10] Senger, L. D., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 326; 1941, **47**, 257.

It is not clear as to why the pituitary gonadotrophin content remains the same during periods of starvation while pituitary lactogen becomes reduced. There can be no doubt that starvation, depending on its degree, results either in a reduction or complete cessation of gonadal and mammary gland activity. This would certainly seem to indicate that the amounts of circulating gonadotrophic and lactogenic hormones are reduced.

On the assumption that pituitary content of a hormone represents the resultant between the amount being produced and the amount released into the blood stream, Maddock and Heller[10] explain the 'dichotomy' between gonadotrophin content in the pituitary and blood stream of starved rats as being due to a failure of release and to a minimum of production by the pituitary. The decrease of lactogenic hormone content in the pituitary during starvation could be explained as being due primarily to a failure of production with little or no interference with the release mechanism.

Assays of pituitary content of gonadotrophin have proven to be reliable indicators of the amounts present in the circulation following ovariectomy, during estrogen administration, but not during starvation. Assays of the lactogen content of the pituitary have accurately reflected the amounts present in the blood stream during lactation, during estrogen or testosterone administration, and also during periods of restricted feed intake.

Summary. The lactogenic and gonadotrophic hormone content of pituitaries of intact and ovariectomized rats was determined after full, ¾, ½, ¼ and no-feed regimes. The full or partially-fed groups were sacrificed at the end of 14 days and the unfed group at the end of 7 days. Pituitary lactogen content was reduced in the intact and ovariectomized rats on the ½, ¼ and no-feed regimes below that of the ¾ and full-fed controls. No change occurred in the gonadotrophin content of the pituitaries of either the intact or ovariectomized rats, regardless of the level of feed intake. In the intact but not in the ovariectomized rats, restricted feed intake caused a marked reduction in the weights of the pituitary, thyroid and adrenals, except that on the no-feed regime adrenal weight was increased in both groups.

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Assay of Secretin and Cholecystokinin Concentrates.*

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The most feasible assay of secretin concentrates involves the cannulation of the pancreatic duct and observation of the increased rate of pancreatic juice output upon intravenous injection of the sample. Ivy, Kloster, Drewyer and Lueth[1] introduced the threshold dose, namely, that amount of a secretin concentrate causing a flow of 10 drops (0.4 ml) of pancreatic juice within a 10-minute period over the control rate in the dog. In a later work, Greengard and Ivy[2] reported that the threshold dose of a preparation obtained from hog duodenums (SI) and adopted as a standard was 0.25 mg for many dogs. This

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[1] Ivy, A. C., Kloster, G., Drewyer, G. E., and Lueth, H. C., *Am. J. Physiol.*, 1930, **95**, 37.

[2] Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 427.

TABLE I.
Assay Values for Secretin Preparations.

	Dog No.	Preparation, mg	SI standard, mg	Pancreatic juice, drops	Secretin potency ratio (units secretin/mg)
Prep. S-61	16	1.0	2.5	12	2.5
	17	1.3	3.0	9	2.3
	51	.81	2.0	28	2.5
	57A	.46	1.1	11	2.4
	58	.75	1.8	38	2.4
	59	1.3	3.2	37	2.5
	67	1.25	3.0	11	2.4
Prep. S-14	1	2.0	3.0	10	1.5
	16	1.5	2.5	9	1.7
	17	2.7	4.0	10	1.5
	25	3.1	4.5	14	1.5
	47	1.95	2.9	10	1.5
	48	2.8	4.2	25	1.5
	49	1.25	2.0	14	1.6
	51	1.3	2.0	15	1.5
	58	1.2	1.8	27	1.5
	61	4.0	6.0	11	1.5
Prep. S-75	1	1.5	3.0	10	2.0
	12	5.0	10.5	9	2.1
	47	1.3	2.6	14	2.0
	51	1.0	2.0	25	2.0
	54	.60	1.2	9	2.0
	55	1.2	2.4	10	2.0
	56	.50	1.05	40	2.1
	57A	.55	1.1	16	2.0
	58	.90	1.8	27	2.0
	59	1.6	3.2	50	2.0
Prep. S-73	12	2.0	10.5	10	5.3
	25	.80	4.5	19	5.6
	26	.95	5.0	10	5.3
	40	.075	0.40	14	5.3
	41	.28	1.5	17	5.4
	47	.50	2.7	8	5.4
	51	.38	2.0	12	5.3
	59	.60	3.2	40	5.3
	64	.56	3.0	40	5.4
	65	.40	2.2	30	5.5
	67	.50	2.8	21	5.6
Prep. S-165	26	.39	5.0	12	13
	36	.32	4.0	9	12.5
	41	.080	1.0	9	12.5
	53	.12	1.5	13	12.5
	58	.14	1.8	27	13
	68	.16	2.0	28	12.5
	71	.20	2.5	33	12.5
	72	.23	3.0	10	13
	73	.25	3.0	17	12
	74	.40	4.8	27	12
Prep. S-36	66	.25	7.6	15	30
	67	.10	3.0	11	30
	70	.12	3.6	20	30
	72	.10	3.0	10	30
	73	.10	3.0	19	30
	76	.10	3.0	32	30
	77	.10	3.0	20	30
	83	.075	2.2	12	29

determined. Further samples may be assayed with frequent checking of the standard dosage.

For the assay of cholecystokinin, the cystic duct is clamped or ligated after isolating it from the cystic artery, and a grooved metal cannula is secured in the gall bladder. A glass tube of 8 mm outside diameter is connected between the cannula and the recording tambour. Where an insufficient amount of fluid is present in the glass tube, additional bile is added. The standard SI is injected and the gall bladder contraction, which is complete within the first few minutes, is recorded in millimeters by noting the initial and final bile levels in the glass tube. When virtually the same initial level is restored, the test extract is introduced in such dosage that the same rise is obtained within 1 ml; however, for a rise of 10 mm or more, 2 mm are often allowed in the comparison, depending on the sensitivity of the gall bladder. The ratio of standard to test dosages is then determined. Since the response to the standard often varies with time and the condition of the dog, frequent or alternate checking is necessary.

It is important in the comparison of a test sample with the standard to treat both in a similar manner especially as regards the initial bile-pressure level, and readings must be taken with consistency particularly in the case of hypersensitive gall gladder preparations. For assay purposes, it was found that more accurate results are obtained by measuring the liquid rise in the glass tube than by comparing the respective heights on the tambour tracing. Secretin and CCK assay values can be checked by employing a comparison at a different drop rate or gall bladder contraction, respectively. The use of derived standards or concentrates of known potency repeatedly checked against the SI standard is also of value.

Since the presence of vasodilatins in a concentrate can have a marked effect on the secretin and CCK assay results, the blood pressure recording must be closely followed. Where additional pentobarbital is administered, sufficient time should elapse before the animal is restandardized.

Variation of Gall Bladder Response with CCK Dosage. A study of the response of the gall bladder to varying doses of CCK concentrates was carried out on several dogs. For some of the large contractions, time intervals up to 45 minutes were required before initial conditions were restored. The majority of the data was rechecked.

Results. Sample data typical for the secretin assay are shown in Table I for 6 preparations carried out on a number of dogs. The respective ratios did not change on comparison of the standard and the test concentrate at a different common drop rate. The value of a respective potency ratio is taken as the number of units of secretin contained in 1 mg of the concentrate. Table II illustrates the potency ratios or CCK unitage per mg for 5 fractions with good reproducibility in almost all cases. The concentration of the standard SI solution was usually 1 mg per ml. Although more potent concentrates were diluted so that the injected volumes of test and standard solutions were similar in many cases, no significant deviations resulted when they differed.

CCK Dose-Response Relationships. By plotting the respective responses or gall bladder contractions against gradually increasing doses of CCK, curves were obtained of the type given in Fig. 1. Except for curve III obtained with a concentrate of 2.0 units of CCK per mg, standard SI was employed.

Discussion. The results of the assay methods outlined for secretin and CCK show clearly that reproducible ratios are obtained which are independent of the weight of the test animal. Furthermore, where the condition of the dog may change after the determination of a ratio, restandardization often gives the same value. This discovery renders the determination of an Ivy secretin threshold dose less reliable than the method herein described. As can be seen from the fluctuation of dosage for a given drop rate from one dog to another in Table I, the former method of assay required at least 10 dogs for an acceptable assay.

The values of the potency ratios are equated to unitage of secretin or CCK contained in 1 mg of the concentrate. The results could be equally well expressed in multiples of these

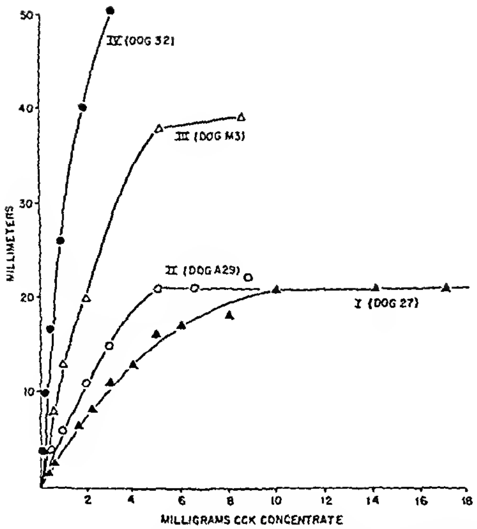

FIG. 1.

Response of the gall bladder to varying doses of cholecystokinin. Curves I, II and IV represent Standard SI; III was obtained for Preparation C-7 containing 2.0 units CCK/mg.

As might be predicted, the administration of increasing doses of CCK brings about gall bladder contractions which increase until a maximum is attained, and beyond which there is no further increase in response in spite of further doses of CCK. This maximum contraction which corresponds to complete emptying of the viscus, varies from one dog to another and is definitely affected by the volume of the gall bladder. Actually, in the CCK assay, volume changes are involved and well-filled gall bladders are preferable. In the comparison of the test sample with the standard, a contraction lower than the maximum must be employed, and this can be checked by noting if a greater bile-pressure rise occurs upon increasing this dosage. Large contractions are avoided, since often a longer time is required for the attainment of initial conditions.

The dose response curve for secretin has been shown by Greengard, Stein and Ivy[11] to be S-shaped in character, and Burn and Holton[12] have used the lower linear portions for the comparison of secretin concentrates with their standard which is comprised of an acetone-dried duodenal powder. Since a maximum in drop response is obtained with elevated doses of secretin, comparisons must be made at drop counts below this limiting value. It should also be pointed out that the log dose-response curves, which are linear for CCK and secretin in the region far removed from their respective maxima, could also be applied to the assay of concentrates. However, it is relatively simple to determine the requisite potency ratio in subsequent dogs by the method outlined in this paper after a first value has been obtained. Usually 3 dogs are sufficient as can be noted in Tables I and II.

Summary. The assay of secretin and CCK concentrates are affected by cannulation of the pancreatic duct and the gall bladder, respectively. The ratio of dosages of a vasodilatin-free standard concentrate (SI) to the test fraction yielding the same number of drops of pancreatic juice in a 10-minute interval is determined, and the value is taken as the secretin unitage contained in 1 mg of test fraction. Similarly, the dosage ratio of this standard to the unknown giving the same gall bladder contraction is equated to the units of CCK per mg of unknown. Good reproducibility is obtained in a large number of dogs.

The dose-response curves for CCK and their significance are discussed.

The authors are grateful to Dr. R. W. Denton for aid in surgical techniques, and to Mr. Max Krup for independent checking of a number of the assays.

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[11] Greengard, H., Stein, I. F., Jr., and Ivy, A. C., *Am. J. Physiol.*, 1941, **132**, 305.

[12] Burn, J. H., and Holton, P., *J. Physiol.*, 1948, **107**, 449.

TABLE II.
Assay Values for CCK Concentrates Compared Against Standard SI.

	Dog No.	Preparation, mg	SI standard mg	Gall bladder contraction, mm	CCK potency ratio (units CCK/mg)
Prep. C-4	16	4.0	6.5	10	1.6
	25	3.1	4.5	11	1.5
	40	.50	.75	12	1.5
	47	1.9	2.4	8	1.3
	48	2.7	4.2	7	1.6
	49	1.25	2.0	15	1.6
	50	1.25	2.0	8	1.6
	51	1.3	2.0	10	1.5
	58	1.2	1.8	23	1.5
	61	4.0	6.0	6	1.5
Prep. C-7	9	4.5	9.0	9	2.0
	37	8.0	16.5	9	2.1
	47	1.3	2.6	9	2.0
	49	.40	.80	8	2.0
	51	1.0	2.0	10	2.0
	54	.60	1.2	10	2.0
	55	1.2	2.4	10	2.0
	56	.50	1.05	8	2.1
	57A	.55	1.1	5	2.0
	58	.90	1.8	20	2.0
Prep. C-60	15	3.5	9.0	10	2.6
	50	.84	2.3	5	2.7
	51	.75	2.0	10	2.7
	56	.39	1.0	7	2.6
	58	.73	1.8	24	2.5
	67	1.25	3.0	20	2.4
Prep. C-37	12	1.2	5.0	10	4.2
	16	1.5	6.5	10	4.3
	25	.90	4.5	11	5.0
	40	.090	.40	10	4.4
	47	.50	2.2	7	4.4
	51	.46	2.0	9	4.3
	64	.70	3.0	11	4.3
	67	.70	3.0	17	4.3
	68	.70	3.0	19	4.3
Prep. C-100P	152	.30	3.5	11	12
	161	.46	5.5	10	12
	170	.60	7.5	35	12.5
	175	.20	2.5	22	12.5

values or in terms of any arbitrary unitage or percentage. Although comparison of test fractions can also be affected by reference to more potent standards, SI has been chosen on the basis of its uniformity of preparation and the presence of both secretin and CCK.

Any method for the biological assay of secretin is fraught with difficulties. For unknown causes, some dogs are very insensitive to secretin. In some cases, after a small dose of secretin is injected the increase in the flow of juice is sustained for longer than 10 minutes. Also, in the case of the CCK assay, it must be remembered that a smooth muscle response is involved. Aside from difficulties arising from the inability of separating the cystic artery and trauma to the gall bladder, some preparations are quite insensitive and, in some cases, the initial conditions of intra-gall bladder pressure are not attained. Only well-functioning gall bladder preparations should be employed for the assay of concentrates. A large number of these difficulties are minimized by employing the present method of comparing the response of test concentrate with that of the standard.

TABLE I.
Effect of Addition of Various Compounds on Anaerobic Growth of *E. coli* in the Absence of CO_2.

C_3-Compounds	Growth	C_4-Compounds	Growth	C_5-Compounds	Growth	C_6-Compounds	Growth
dl-Alanine	6	Succinic Acid	30	α-Ketoglutaric Acid	85	Citric Acid	9
Pyruvic Acid	4	Fumaric Acid	40	Glutamic Acid	200	cis-Aconitic Acid	25
		Malic Acid	35				
		Oxalacetic Acid	220				
		Aspartic Acid	62				

All compounds were used in 2-mM quantities. Growth is indicated by turbidimetric readings on the photoelectric colorimeter.
Control experiments give readings from 3 to 9.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C

TABLE II.
Effect of Added Carbonate on the Growth of *E. coli* in the Presence of C_4 Compounds.

Compound	With $NaHCO_3$	Without $NaHCO_3$
Succinic	55	30
Fumaric	65	40
Malic.	60	45

All of the compounds including $NaHCO_3$ were added in 2-mM quantities. The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.

Control gave readings from 3 to 9.

Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

in the place of CO_2 (Table IV). *E. coli* will not utilize citric acid under the above conditions.

The fact that the compounds normally occurring in the Krebs cycle replace CO_2 anaerobically suggests a possible function of this cycle in the absence as well as in the presence of oxygen. However, it should be noted that the existence of this cycle has not been demonstrated in bacterial respiration.

It is generally considered that the tricarboxylic acid cycle is an aerobic mechanism by which various substrates can be oxidized to CO_2 and H_2O, to yield energy to the system. Since the final products of anaerobiosis are not generally the same as in the presence of O_2, some other function of this cycle must be proposed. Since fat, protein, and carbohydrate metabolism have many points in common in the form of identical intermediate products and since the compounds supplied in the absence of CO_2 are some of the interconvertible intermediate products, it is possible that by amination, transamination, and other reactions the various compounds are directly utilized for the synthesis of proteins. On such a basis the utilization of CO_2 to form these compounds which in turn are used for cellular material can then be explained.

Summary. A number of compounds have been found which will replace CO_2, necessary for the anaerobic metabolism of *Escherichia coli* and *Aerobacter aerogenes*. The C_5 compounds appear to replace CO_2 more effectively than other compounds tested with the exception of oxalacetic acid and citric acid, the latter in the case of *A. aerogenes* only. The

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Anaerobic Replacement of Carbon Dioxide.*

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Previous reports[1] on the heterotrophic replacement of carbon dioxide in bacteria have dealt exclusively with aerobic metabolism, largely because the compounds substituting for CO_2 were known to occur in the normal aerobic metabolism of bacteria. Further research in this field, however, reveals first that organisms normally requiring CO_2 when growing aerobically require it anaerobically, and secondly, that the same compounds replace CO_2 under anaerobic conditions. The purpose of the present communication is to report on the anaerobic replacement of carbon dioxide and to compare the function of these compounds under aerobic and anaerobic conditions.

Methods. The methods used were essentially those employed in previous work.[1] The organisms used in these experiments were *Escherichia coli* and *Aerobacter aerogenes.* The inoculum consisted of a 24-hour culture grown in 10 ml of the basal medium consisting of 0.8% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, 0.8% glucose and 10% tap water, final pH adjusted to 6.8. The compounds tested were added before autoclaving except in the case of oxalacetic acid. A solution of the sodium salt was sterilized by filtration and added aseptically.

Cylinder nitrogen was passed over hot reduced copper gauze to remove oxygen and then through a CO_2-absorbing train before passing through the cultures. The reaction flask contained 25 ml of basal medium to which 2% of inoculum was added aseptically. Growth was measured by turbidimetric readings on a Klett-Summerson photoelectric colorimeter with a 660 mμ light filter. All readings were made after 12 hours incubation at 37°C. Sterile, uninoculated media were used as blanks.

Experimental. Neither *E. coli* nor *A. aerogenes* will grow anaerobically in the absence of CO_2. Atmospheric CO_2 can be replaced by $NaHCO_3$; these results are in accord with Gladstone *et al.*[2] Since no growth takes place in the absence of CO_2, the conclusion may be drawn that CO_2 has a definite function under anaerobic conditions.

The compounds and the extent to which they replace CO_2 are listed in Table I. The results are essentially similar to those obtained aerobically with the exception of oxalacetic acid which most effectively replaces *CO_2 in the complete absence of oxygen. It is,* therefore, possible that oxalacetic acid is a key compound in the anaerobic metabolism of the cells.

α-Ketoglutaric acid (and glutamic acid) substitutes anaerobically to a greater extent than any of the other compounds, except oxalacetate. Similar results were obtained aerobically. These facts again suggest a possible further fixation over and above the Wood and Werkman reaction. Such a fixation reaction would in normal metabolism (in the presence of CO_2) yield C_5 acids which are more essential to the cell. Indirect evidence for this has been obtained. More abundant growth is obtained with bicarbonate and succinic, malic or fumaric acid than with any of these acids alone (Table II).

A number of compounds metabolically related to those of Table II will also replace CO_2 anaerobically (Table III).

In the case of *A. aerogenes,* the function of citric acid under anaerobic conditions should be stressed. Whereas, the effect is comparatively small aerobically, in the absence of O_2 the organism uses this acid very effectively

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* Journal Paper No. J-1199 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 862.

[1] Ajl, S. J., and Werkman, C. H., *Arch. Biochem.*, 1948, **19**, 483.

[2] Gladstone, G. P., Fildes, P., and Richardson, G. M., *Brit. J. Exp. Path.*, 1935, **16**, 335.

TABLE I.
Hyaluronidase Inhibitor Activity of Human Blood Serum Fractions in the Presence of Different Amounts of Magnesium Ion.

	No added magnesium		Additional magnesium	
	mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum	mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum
Serum A	4.4	70	68	150
Component IA	.8	7	60	28
” IIA	.8	5	60	75
” IA and IIA	.8	10	60	100
Residue	2.9	—	—	—

inhibitor may be a polysaccharide or protein-polysaccharide complex successfully competing with the hyaluronic acid as an alternate substrate.[3,7,12]

Partial separation of serum into two fractions that were inactive alone, but active when recombined, seemed to indicate that the inhibition was probably more complicated than originally supposed. Both fractions were first reported to be of protein nature:[9] Component I was flocculated from serum at -2°C, pH 8.4, and 25% ethanol; followed by component II flocculated by lowering the pH to 6.1. Another report[10] showed that citrated, oxalated, and dialyzed serum was not active unless magnesium was added. Studies in these laboratories indicated that when human blood serum was fractionated according to method of Cohn *et al.*,[17] much of the activity appeared in fractions II and III; but only if the fraction was dissolved in a solution containing magnesium ions.

Further experiments on the fractionation of normal human serum with respect to the hyaluronidase inhibitor and the contribution of magnesium to the activity of the inhibitor are herewith reported.

Methods. Twenty frozen stored samples of human blood sera were selected at random from a series originally obtained from young adult males. The hyaluronidase, from frozen bovine testis, was prepared, assayed, and standardized by previously described methods.[18,19] Sera were assayed for hyaluronidase inhibitor by incubating suitable dilutions with standardized hyaluronidase, subsequently assaying the reaction mixture for active hyaluronidase.[12] Dilutions were made with saline-borate buffers with and without measured amounts of magnesium chloride. Magnesium content of the undiluted serum and its fractions was determined with titan yellow.[20] Thus, the relative amounts of serum and magnesium present in the reaction mixture could be calculated and the relation expressed as millimoles of magnesium per ml of reaction mixture. The inhibitor activity was calculated as units of hyaluronidase inactivated per ml of serum.[19] Increased amounts of magnesium were added to diluted samples of serum until 4 or 5 successively higher magnesium concentrations gave no further increase in inhibitor activity.

Normal human serum was buffered to pH 8.4 with barbiturate, cooled to 0°C, and component I was flocculated by the slow addition of an equal volume of 50% ethanol. After separating and washing the precipitate in a refrigerated centrifuge, the supernate was brought to pH 6.1 with 0.6 *M* acetate buffer, pH 4.0. The precipitate that formed, component II, was removed and washed in refrigerated centrifuge. The two precipitates were suspended in volumes of 0.15 *M* saline equal to the volume of the original serum and

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[17] Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, N. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

[18] Hahn, L., *Ark. Kemi. Mineral O. Geol.*, 1944, **19A**, No. 33.

[19] Dorfman, A., and Ott, M. L., *J. Biol. Chem.*, 1948, **172**, 367.

[20] Kunkel, H. O., Pearson, P. B., and Schweigert, B. S., *J. Lab. and Clin. Med.*, 1947, **32**, 1027.

TABLE III.
Effect of Glutamine, Asparagine, Arginine, and Proline on Anaerobic Growth of *E. coli* in the Absence of CO_2.

Compound	Glutamine	Asparagine	Arginine	Proline	Control
Growth	125	40	55	30	5

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.
Compounds were added in 2-mM quantities.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

TABLE IV.
Replacement of CO_2 by Citric Acid Under Aerobic and Anaerobic Conditions.

Organism	Citric Acid	
	Nitrogen with O_2 and CO_2 removed	Air with CO_2 removed
Escherichia coli	9	4
Aerobacter aerogenes	180	50

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.
2-mM quantities of citric acid were used.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

fact that oxalacetic by-passes the CO_2 requirement to such an extent indicates that this acid may function as the chief substrate for protein synthesis under anaerobic conditions.

16979

Magnesium and Hyaluronidase Inhibitor of Blood Serum.

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Numerous reports[1-12] have established the presence of a hyaluronidase inhibitor in the blood sera of several species. The nature of the inhibitor has not been elucidated: but evidence has been presented that it is not a specific antibody;[2,13-16] nor an enzyme specifically attacking hyaluronidase.[6,9,12] The

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[*] Address: Department of Pediatrics, University of Chicago Medical School.

[†] Contribution from University of Chicago carried out under contract with Medical Research and Development Board, Surgeon General, U. S. Army.

[1] Duran-Reynals, F., *J. Exp. Med.*, 1933, **58**, 161.

[2] McClean, D., *J. Path. and Bact.*, 1936, **42**, 477.

[3] McClean, D., *J. Path. and Bact.*, 1942, **54**, 284.

[4] McClean, D., *Biochem. J.*, 1943, **37**, 169.

[5] Leonard, S. L., and Kurzok, R., *Endocrinol.*, 1945, **37**, 171.

[6] Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

[7] Dorfman, A., Ott, M. L., and Reimers, E., *J. Fed. Proc.*, 1947, **6**, 248.

[8] Hechter, O., and Scully, E. L., *J. Exp. Med.*, 1947, **86**, 19.

[9] Goldberg, A., and Haas, E., *J. Biol. Chem.*, 1947, **170**, 757.

[10] Bamberger, J. P., and Fried, Naomi, *J. Biol. Chem.*, 1948, **172**, 347.

[11] Hadidian, Z., and Pirie, N. W., *Biochem. J.*, 1948, **42**, 266.

[12] Dorfman, A., Ott, M. L., and Whitney, R., *J. Biol. Chem.*, 1948, **174**, 621.

[13] McClean, D., and Hale, C. W., *Biochem. J.*, 1941, **35**, 159.

[14] Duran-Reynals, F., *J. Exp. Med.*, 1932, **58**, 703.

[15] Duran-Reynals, F., *J. Exp. Med.*, 1939, **69**, 69.

[16] Hobby, G. L., Dawson, M. H., Meyer, K., and Chaffee, E., *J. Exp. Med.*, 1941, **73**, 109.

TABLE II.
Hyaluronidase Inhibitor Activity of Blood Sera With and Without Added Magnesium.

	Normal blood serum		Added magnesium	
Sample	mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum	Total mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum
925	5.0	43	65	75
920	3.5	24	66	87
917	4.1	40	68	110
929	4.1	50	67	112
918	4.1	50	68	116
916	5.2	47	68	117
934	4.1	73	67	145
919	3.5	71	68	148
921	3.3	63	68	150
932	4.1	78	67	150
923	4.2	62	68	165
926	4.5	63	68	167
924	5.0	75	68	175

saline dilutions were again very low in magnesium; and consequently, showed little activity. When sufficient magnesium was added, the activities of the mixtures were greatly enhanced.

These experiments clearly indicated that the hyaluronidase inhibitor in human serum and in the active serum fractions required some minimum concentration of magnesium ion for expression of its maximum activity *in vitro*. The actual amounts of magnesium required for maximum activity were determined by experiments illustrated in Fig. 1. These data demonstrated that when sera, or its active fractions, were diluted with saline alone, very low inhibitor activities were found in the assay reaction mixtures. Increasing amounts of magnesium in the reaction mixture were accompanied by regular increments of activity until a maximum activity was reached where the reaction mixture contained about 50 to 60 x 10^{-5} millimoles of magnesium ion per ml. Further increases or decreases in activity were within the experimental error of the assay up to concentrations of 200 x 10^{-5} millimoles.

It should be noted, however, that the assay method employed demanded an inhibitor concentration of only 2 to 4 units per ml in reaction mixture. This restriction necessitated a 20 to 100-fold dilution of most sera. Thus, when the serum was diluted with saline alone, the magnesium concentration, about 80 x 10^{-5} millimoles per ml of serum, fell to less than 5 x 10^{-5} millimoles per ml of the reaction mixture. Consequently, low inhibitor values resulted. Maximum inhibitor values were found when the magnesium concentration was increased to 60 x 10^{-5} millimoles per ml, very near that of the original serum. This was clearly shown in Fig. 1 where data were plotted for component IIB and 4 sera of high, low, and intermediate activity.

Thirteen additional samples were determined by diluting the sera with saline alone, and by diluting with saline containing adequate concentrations of magnesium. These data, showing the necessity of magnesium in the inhibitor assay, are given in Table II.

Summary. Magnesium ion has been found to be essential for the hyaluronidase inhibitor activity of human blood under the conditions of the *in vitro* assay. The minimum amount of magnesium required for expression of maximum activity has been determined for the assay method employed in these laboratories.

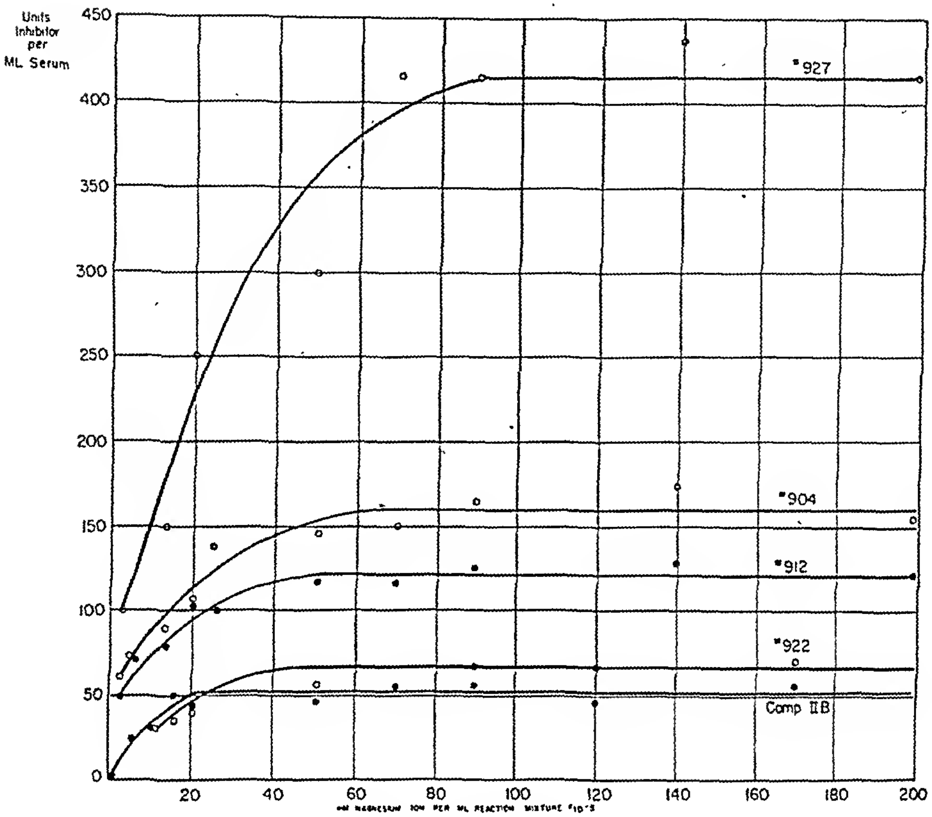

Fig. 1.
Relation of magnesium concentration to inhibitor activity in assay reaction mixture.

assayed for hyaluronidase inhibitor with and without additional magnesium. Serum components and supernate were analyzed for magnesium and assayed for inhibitor with the results shown in Table I as units of inhibitor per ml of serum and millimoles of magnesium per ml of the reaction mixture.

Table I illustrates the values found in 3 fractionations of normal human sera. The original sera contained approximately 80.10^{-5} millimoles of magnesium ion per ml. This is 18 times the magnesium ion content of the assay reaction mixture after suitable dilution of the serum with saline. This reaction mixture assayed 70 inhibitor units per ml of serum. In order to obtain the maximum inhibitor activity of 150 units per ml of serum, it was necessary to increase the magnesium concentration of the assay mixture to at least $60 + 10^{-5}$ millimoles per ml by diluting the serum with saline-magnesium solutions instead of saline alone.

The washed precipitates of component I contained small amounts of magnesium: less than one-fifth of the total serum magnesium. It was apparent that the precipitates contained insufficient magnesium when suitably diluted with saline for assay. When these precipitates were diluted with saline containing sufficient magnesium to support maximum inhibition, only a part of the total inhibitor was found.

Component II precipitates also contained minimum amounts of magnesium and when diluted with saline alone gave little indication of inhibitor activity. When diluted with saline-magnesium solution, *however, these* precipitates gave inhibitor values equal to at least half of the total activity of the serum. When components I and II were mixed, the

TABLE I.
Lack of Effect of Darvisul on MM Virus and Lansing Poliomyelitis Virus Infection in Mice.

Exp. No.		Darvisul		Infection										
		Dose, mg/day	Drug route	Virus route	Virus Strain	Conc. of virus							LD$_{50}$ titer	Remarks
						10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
1	Control	0	—	IP	MM			15/15*	15/15	14/14	11/12		$10^{-6.5}$ or more	Drug admin. begun 24 hr after infection and continued 5 days.
	Treated	16	IP	,,	,,			15/15	14/15	13/13	9/14		$10^{-6.2}$ or more	
2	Control	0	—	,,	,,				10/10	9/10	4/10		$10^{-5.7}$	Drug admin. 2 days pre and 6 days post infection.
	Treated	16	IP	,,	,,				7/9	2/10	1/9		$10^{-4.6}$	
3	Control	0	—	,,	,,				10/10	7/10	2/10	1/10	$10^{-5.5}$	Ditto No. 2.
	Treated	16	IP	,,	,,			10/10	10/10	7/10	5/10	1/10	$10^{-5.8}$	
4	Control	0	—	SC	,,					9/9	8/10	4/10	$10^{-6.7}$	Ditto No. 2.
	Treated	16	IP	,,	,,					9/9	7/10	2/9	$10^{-6.1}$	
5	Control	0	—	oral	,,		4/9	1/8						Ditto No. 2.
	Treated	16	IP	,,	,,		6/10	6/10						
6	Control	*In vitro* exper.		IP	,,		6/6	6/6	4/6	0/6	0/6		$10^{-4.2}$	1% virus vs. 1% drug at 37°C for 2 hr after which dilutions were made with distilled water.
	Treated			,,	,,		6/6	6/6	6/6	6/6	5/6		$10^{-6.4}$ or more	
7	Control	0	—	IC	LA	7/8	4/8	0/8	0/8				$10^{-1.7}$	Ditto No. 2.
	Treated	16	IP	,,	,,	7/7	2/8	0/8	0/8				$10^{-1.7}$	

* 15/15 Numerator = number of mice paralyzed; denominator = number of mice inoculated. Mice dying without symptoms have been excluded. IP = Intraperitoneal; IC = Intracerebral; SC = Subcutaneous; LA = Lansing poliomyelitis virus.

TABLE II.
Lack of Effect of Darvisul on the Excretion of Theiler's Virus (TO).

Exp. No.	Darvisul		Assay of TO virus in feces of individual mice	
	Individual mice treated	Average dose drug/day, mg	Before Darvisul	After 7 days of Darvisul
8	Mouse No. 1	88	8/10*	8/10
	,, ,, 2	74	2/9	1/7
	,, ,, 3	72	10/10	9/9

* 8/10 = 8 out of 10 mice became paralyzed following inoculation of feces from mouse No. 1.

16980

Lack of Effect of Sodium Phenosulfazole (Darvisul) on Certain Experimental Virus Infections.*

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Sodium phenosulfazole, or Darvisul, (N-(2-thiazolyl)-phenol sulfonamide)† has recently been described by Sanders, SubbaRow and Alexander[1] as protecting mice against as many as 100 LD_{50} doses of the Columbia SK murine virus.

The present report is concerned with tests of Darvisul against the MM virus,‡ the Lansing strain of poliomyelitis virus, and Theiler's virus of mouse encephalomyelitis (TO strain). No protective effect was found.

CFW mice (Carworth Farms) 5 to 6 weeks old, weighing 15 to 20 g were used in all but one experiment. Eight mg of Darvisul in 1 ml of physiologic saline were given twice daily by the intra-abdominal route. In the experiment on Theiler's virus (Table II) 3 to 4 week old mice were used conforming to our standard procedure with TO virus and the drug was fed in the diet.

In Experiment 1 (Table I) the procedure described by Sanders and his associates[1] was followed precisely, *e.g.* 8 mg of Davisul were given twice daily beginning 24 hours after intra-abdominal injection of MM virus and continued for 5 days. No significant difference was observed in the treated and control animals. In Experiments 2 and 3, Darvisul was begun 2 days before and continued for 6 days after infection. The suggestive difference obtained in Experiment 2 was not reproduced in Experiment 3. In Experiments 4 and 5, MM virus was given by a route different from that of Darvisul—subcutaneously and by mouth, respectively. No appreciable effect was noted. The *in vitro* effect of Darvisul on MM virus was tested in Experiment 6. Instead of inactivation of virus, the titer of the suspension of virus plus Darvisul was more than 100 times greater than the titer of the virus control. In Experiment 7, Darvisul was begun 2 days before and continued for 6 days after intracerebral injection of the Lansing strain of poliomyelitis virus. The results were negative. Experiment 8 is concerned with Theiler's virus of mouse encephalomyelitis as found in the intestines of normal mice (Table II). The capacity of Darvisul to reduce the amount of TO virus excreted in the feces was tested. The stools of individual mice were assayed for virus before and after drug administration. In this experiment a 2% drug diet was fed to 3 mice for 7 days. The amount of Darvisul consumed was 72 to 88 mg per day. In contrast to certain other compounds tested in this laboratory,[4] Darvisul had no effect upon the excretion of TO virus in the feces of these mice.

Summary. Under the circumstances which have been described, Darvisul revealed neither prophylactic nor therapeutic value in mice infected with the MM virus, the Lansing strain of poliomyelitis virus or Theiler's virus of mouse encephalomyelitis (TO).

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

† This compound was made available through the courtesy of Lederle Laboratories Division, American Cyanamide Co., Pearl River, N. Y.

[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Reports on Biology and Medicine*, 1948, 6, 385.

‡ MM virus has been shown to be closely related serologically to Columbia SK virus and the virus of encephalomyo-carditis.[2,3]

[2] Warren, J., and Smadel, J. E., *Fed. Proc.*, 1948, 7, 311.

[3] Ward, R., and Rader, D. L., unpublished work.

[4] LoGrippo, G. A., Earle, D. P., Jr., Brodie, B. B., Graef, I. P., Bowman, R. L., and Ward, R., unpublished work.

TABLE I.
Lack of Effect of Darvisul on MM Virus and Lansing Poliomyelitis Virus Infection in Mice.

Exp. No.		Darvisul		Infection										
						Conc. of virus								
		Dose, mg/day	Drug route	Virus route	Virus Strain	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	LD_{50} titer	Remarks
1	Control	0	—	IP	MM			15/15*	15/15	14/14	11/12		$10^{-6.5}$ or more	Drug admin. begun 24 hr after infection and continued 5 days.
	Treated	16	IP	,,	,,			15/15	14/15	13/13	9/14		$10^{-6.2}$ or more	,, ,,
2	Control	0	—	,,	,,				10/10	9/10	4/10		$10^{-5.7}$	Drug admin. 2 days pre and 6 days post infection.
	Treated	16	IP	,,	,,				7/9	2/10	1/9		$10^{-4.6}$	
3	Control	0	—	,,	,,				10/10	7/10	2/10	1/10	$10^{-5.5}$	Ditto No. 2.
	Treated	16	IP	,,	,,			10/10	10/10	7/10	5/10	1/10	$10^{-5.8}$	
4	Control	0	—	SC	,,					9/9	8/10	4/10	$10^{-6.7}$	Ditto No. 2.
	Treated	16	IP	,,	,,					9/9	7/10	2/9	$10^{-6.1}$	
5	Control	0	—	oral	,,		4/9	1/8						Ditto No. 2.
	Treated	16	IP	,,	,,		6/10	6/10						
6	Control	*In vitro* exper.		IP	,,		6/6	6/6	4/6	0/6	0/6		$10^{-4.2}$	1% virus vs. 1% drug at 37°C for 2 hr after which dilutions were made with distilled water.
	Treated			,,	,,		6/6	6/6	6/6	6/6	5/6		$10^{-6.4}$ or more	
7	Control	0	—	IC	LA	7/8	4/8	0/8	0/8				$10^{-1.7}$	Ditto No. 2.
	Treated	16	IP	,,	,,	7/7	2/8	0/8	0/8				$10^{-1.7}$	

* 15/15 Numerator = number of mice paralyzed; denominator = number of mice inoculated. Mice dying without symptoms have been excluded. IP = Intraperitoneal; IC = Intracerebral; SC = Subcutaneous; LA = Lansing poliomyelitis virus.

TABLE II.
Lack of Effect of Darvisul on the Excretion of Theiler's Virus (TO).

Exp. No.	Darvisul		Assay of TO virus in feces of individual mice	
	Individual mice treated	Average dose drug/day, mg	Before Darvisul	After 7 days of Darvisul
8	Mouse No. 1	88	8/10*	8/10
	,, ,, 2	74	2/9	1/7
	,, ,, 3	72	10/10	9/9

* 8/10 = 8 out of 10 mice became paralyzed following inoculation of feces from mouse No. 1.

16981

Failure of Phenosulfazole (Darvisul) in Treating Experimental Viral Infections.

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Recently Sanders, SubbaRow and Alexander[1] reported sodium phenosulfazole (Darvisul), N-(2-thiazolyl)-phenol sulfonamide, to be effective against at least as many as 100 LD_{50} doses of the Columbia SK virus[2,3] for mice when intraperitoneal treatment was instituted 24 hours after intraperitoneal infection.

The purpose of this communication is to report the failure of phenosulfazole to protect mice and monkeys against experimental infection with the Columbia SK virus, and the Brunhilde strain of poliomyelitis virus, respectively, as well as its lack of effect against certain other viral infections in mice and in chick embryos.

A. Experiments with Columbia SK Virus. Exp. 1. Groups of 15 mice (Rockland Swiss albino), weighing 20 to 24 g each, were inoculated intraperitoneally with graded doses of Columbia SK virus (a frozen pool of infected mouse brains received through the courtesy of Dr. Murray Sanders) and treated with phenosulfazole by the same route. Treatment was started 24 hours after virus inoculation and continued for 6 consecutive days. The phenosulfazole used in this experiment was obtained from Dr. Sanders as a 25% solution and represented a portion of the same preparation that was used in his laboratory. The drug was diluted with sterile saline solution to give a concentration of 8.26 mg per ml and given at 8 and 11:30 a. m., and 3 and 5 or 6 p. m. in doses of 0.5, 0.25, 0.25 and 1.0 ml respectively. Thus, the dose per day per mouse was 16.5 mg. Control mice were injected in a manner similar to the treated group except that sterile physiological saline solution was used. The mice were observed for 21 days at which time the mortality ratios were calculated.

Exp. 2 This experiment was similar to Exp. 1 except that slightly smaller mice (Rockland Swiss albino), about 18 to 20 g in weight, were used. Dilutions of the Columbia SK virus, 10^{-4} through 10^{-7}, were used with 20 mice per each dilution. Group 1 mice received 22 mg phenosulfazole* per mouse per day. Group 2 mice received 32 mg of drug per mouse per day. Group 3 mice served as virus controls and received injections of sterile saline solution in place of the drug. Group 4 mice received no virus but served as drug controls and received 22 mg phenosulfazole per day. Group 5 (only 10 mice in this group) likewise received no virus but received 32 mg phenosulfazole per day as drug controls.

Treatment was started 20 hours after infection and continued for 12 days. The drug was used in a concentration of 20 mg per ml. The mice received 4 injections (intraperitoneally) per day. Those of Group 1 and 4 received 6 mg at 8 a. m., 4 mg at 11:30 a. m., and 3 p. m., and 8 mg at 5 p. m., whereas Groups 2 and 5 received 8 mg each at 8 and 11:30 a. m., and 3 and 5 p. m. The mice were

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[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Reports Biol. and Med.*, 1948, **6**, 385.

[2] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

[3] Committee on Nomenclature of the National Foundation for Infantile Paralysis, A Proposed Provisional Definition of Poliomyelitis Virus, *Science*, 1948, **108**, 701.

* The phenosulfazole used in this and most of the subsequent experiments was received from Dr. M. C. Lockhart, Director of the Pharmaceutical Section, Lederle Laboratories Division, American Cyanamide Company, as a sterile 25% solution in sealed glass ampoules.

TABLE I.
Results Obtained with Phenosulfazole in the Treatment of Mice Infected with Columbia SK Virus.

Exp. No.	Wt of mice, g	Drug per day, mg	Mortality ratio† of mice infected with virus dilutions 10^{-4}	10^{-5}	10^{-6}	10^{-7}	LD_{50} titer
1	20-24	16.5	15/15	13/15	9/15	12/15	$10^{-7.0}$
		0* (Virus control)	12/15	12/15	9/15	11/15	$10^{-6.6}$
2	18-20	22	16/20	12/20	4/20	7/20	$10^{-5.4}$
		32	14/19	12/20	7/20	9/20	$10^{-5.6}$
		0* (Virus control)	10/19	9/20	8/20	4/20	$10^{-5.0}$
		22 (No virus drug control)	0/20				
		32 (No virus drug control)	0/10				

* *Received injections of buffered saline solution instead of drug.*

† Numerator = No. of mice dying; denominator = No. of mice in group.

observed for 16 days at which time the mortality ratios were calculated.

The results of the first two experiments are summarized in Table I. The data show that phenosulfazole had no beneficial therapeutic effect in mice infected with the Columbia SK virus and treated as described above. In addition there was no evidence to suggest that the drug affected the onset or duration of the infection.

B. Experiments with Poliomyelitis Virus in Monkeys. Exp. 3. Five monkeys (*M. rhesus*) were infected intranasally under ether anesthesia with 4 ml each of a 10% suspension of a frozen pool of monkey cords infected with the Brunhilde strain of poliomyelitis virus (3rd passage in this laboratory of material received from Dr. Isabel Morgan, Baltimore, Md.). Three monkeys were treated with phenosulfazole whereas 2 served as virus controls and received sterile saline solution in place of the drug. Treatment was started 24 hours after infection and consisted of 200 to 300 mg intravenously at 8 a. m., 300 mg intraperitoneally (intravenously on one occasion) at 12 noon, and 500 mg intraperitoneally at 5 p. m. Treatment was continued for 11 days. The results may be summarized by stating that no evidence was obtained to indicate that treatment with phenosulfazole showed any beneficial effect.

The 3 treated monkeys reacted as follows: No. 26 showed definite weakness of both arms and legs on the 5th day, complete paralysis of both legs on the 8th day, was prostrate on the 9th day, and was sacrificed in a moribund condition on the 10th day. No. 34 showed tremors and weakness of both legs and arms on the 10th and 11th day, paralysis of the left arm and leg on the 12th and 13th day, extreme weakness with inability to move from the 14th to 23rd day but from thereon gradually improved and eventually recovered with some residual paralysis. No. 43 showed progressive weakness from the 11th through the 13th day, was paralyzed in both arms and legs on the 14th day and was sacrificed in a moribund condition on the 15th day.

The 2 untreated monkeys reacted as follows: No. 9 showed no signs of illness throughout the 28 days of observation, whereas No. 42 developed paralysis of both legs and arms on the 15th day and was sacrificed in a moribund condition on the 16th day.

C. Experiments with some Neurotropic Viruses and Influenza Virus in Mice. Three experiments were carried out with the MEF-1 strain of poliomyelitis virus (obtained through the courtesy of Dr. Peter K. Olitsky of the Rockefeller Institute for Medical Research, New York, N. Y.), using 50 to 500 LD_{50} doses inoculated intracerebrally into Rockland Swiss albino mice. In Exp. 1 treatment of the infected animals was initiated 24 hours after infection. In Exp. 2, in which 2 separate groups were treated, drug therapy was started 24 hours after infection in one group, and 6 hours after infection in the other. In Exp. 3, two groups of treated animals were again employed with the drug being administered 6 hours after infection in one group, and 1½ hours after infection in the other.

Two experiments were carried out with Eastern equine encephalomyelitis virus using Tumblebrook Swiss albino mice. In one case the mice were inoculated by the intracerebral route and in the other by the intraperitoneal route. In both cases drug therapy was initiated 24 hours following infection.

Two experiments were carried out using Venezuelan equine encephalomyelitis virus inoculated subcutaneously into Rockland Swiss albino mice. In both experiments drug treatment was started 24 hours following infection.

A single experiment was carried out with Russian Spring-Summer encephalitis virus inoculated subcutaneously into Carworth Swiss albino mice. Drug therapy was started 24 hours following infection.

Two experiments were carried out with the PR8 strain of influenza virus (Type A) inoculated intranasally into Rockland Swiss albino mice. In the first experiment drug therapy was started 24 hours following infection whereas in the second it was started 1½ hours following infection.

The essential data pertaining to these experiments are shown in Table II. The results may be summarized by stating that again no evidence was obtained to show that phenosulfazole had any beneficial therapeutic effect in mice infected with any of the above named viral infections.

D. Experiments in Chick Embryos. A number of experiments were carried out to determine the effect of phenosulfazole upon chick embryos infected with Newcastle disease virus, the Lee strain (Type B) of influenza virus, the Bitter Root strain of Rocky Mountain spotted fever (*R. rickettsii*), the Breinl strain of epidemic or louse-borne typhus (*R. prowazekii*) and the 6 BC strain of psittacosis (*M. psittacii*). The experiments may be summarized by stating that only in the case of psittacosis, in which relatively large doses of the drug were employed (25 mg per embryo), was there any suggestive evidence that phenosulfazole had a protective effect. This was demonstrated by the fact that drug-treated embryos showed a slight increase in their survival time and a reduction in the number of elementary bodies observed by microscopic examination of yolk-sac smears, as compared to infected control eggs treated with saline in a similar manner. This was not an unexpected finding, however, because other sulfonamides, particularly sulfadiazine, have been shown to be active against psittacosis.[4-6] It should be pointed out, however, that the results obtained with phenosulfazole in the treatment of chick embryos infected with *M. psittacii* were not nearly as satisfactory as those reported recently for the antibiotic Aureomycin.[7]

Conclusion. Phenosulfazole (Darvisul) showed no beneficial effect in the treatment of mice infected with the Columbia SK virus and other neurotropic viruses, including a rodent adapted strain of poliomyelitis (MEF-1 strain). The lack of therapeutic effectiveness of the drug was also observed in monkeys infected intranasally with the Brunhilde strain of poliomyelitis virus. In other viral and rickettsial infections of mice and of developing chick embryos treatment with phenosulfazole was of no therapeutic value, with the possible exception of psittacosis in which a slight inhibition of growth in the developing chick embryos was observed. However, even in the latter case the effectiveness of phenosulfazole therapy was of much lower magnitude than that observed with other sulfonamides,[4-6] or antibiotics.[7]

ADDENDUM

Since this paper was written an additional experiment was carried out using Columbia SK virus in Carworth Swiss albino mice in a manner as identical as possible to that em-

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[4] Meiklejohn, G., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 1.

[5] Wiseman, R. W., Meiklejohn, G., Lackman, D. B., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 9.

[6] Rosebury, T., Ellingson, H. V., and Meiklejohn, G., with assistance of Schabel, F., *J. Infect. Dis.*, 1947, **80**, 64.

[7] Wong, S. C., and Cox, H. R., *Ann. N.Y. Acad. Sc.*, 1948, **51**, 290.

TABLE II.

Results Obtained with Phenosulfazole in the Treatment of Mice Infected with Other Viruses.

Virus inoculum	Route of infection	Exp. No.	Wt of mice, g	Drug per day, mg	Mortality ratio* of mice infected with virus dilutions								LD_{50} titer
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Poliomyelitis MEF-1	I.C.	1	10-12	10	10/12								
				0†	6/6								
		2	10-12	16	10/10	8/10							
				16‡	10/10	10/10							
				0†	9/10	9/10							
		3	10-14	8‡	12/12								
				8§	12/12								
				0†	12/14								
Eastern Equine Encephalomyelitis	I.C.	1	14-16	20						6/6	2/5	3/6	$10^{-7.1}$
				0†						6/6	6/6	0/6	$10^{-7.5}$
	I.P.	2	14-16	20				3/6	4/6	1/6	0/6		$10^{-4.7}$
				0†				1/6	1/6	0/6	0/6		$<10^{-4.0}$
Venezuelan Equine Encephalomyelitis	S.C.	1	20-22	4				6/6	6/6	5/5	6/6	6/6	$>10^{-8.0}$
				0†						6/6	0/6	0/6	$10^{-6.5}$
		2	20-22	8				6/6	6/6	5/6	3/6	3/6	$10^{-7.3}$
				0†				6/6	6/6	4/6	1/6	1/6	$10^{-6.1}$
Russian Spring-Summer Encephalitis	S.C.	1	18-20	20			4/4						
				0†‖			4/4						
Influenza PR-8	I.N.	1	16-18	22					8/9				
				0†					8/9				
		2	16-18	22§					9/9				
				0†					8/9				

* Numerator = No. of mice dying; denominator = No. of mice in group.

† Untreated controls.

‡ Drug treatment started 6 hr after virus inoculation.

§ Drug treatment started 1½ hr after virus inoculation.

‖ The 10^{-3} dilution of Russian Spring-Summer encephalitis contained 25 to 100 mouse LD_{50}.

ployed by Sanders *et al.*[1] Groups of 25 to 28 mice, weighing 16-20 g each, were inoculated intraperitoneally with 0.06 ml each of graded doses of SK virus ranging from 2×10^{-4} to 2×10^{-8}. Treatment was initiated 24 hours after virus inoculation and continued for 5 consecutive days. Group 1 mice received 1 ml of the drug intraperitoneally (8.33 mg phenosulfazole per ml) at 8 a.m. and 6 p.m. Group 2 mice were injected in similar manner with sterile saline solution, while Group 3 mice received no treatment. The LD_{50} titers were as follows: Group 1 (phenosulfazole treated) $10^{-6.3}$, Group 2 (saline treated) $10^{-6.6}$, Group 3 (no treatment) $10^{-6.1}$.

16982

Therapeutic Failure of Phenosulfazole (Darvisul) in Mice Infected with EMC or with MM Viruses.

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Phenosulfazole (Darvisul) has been reported by Sanders, SubbaRow, and Alexander[1] as having a therapeutic effect in mice infected with "a mouse virus isolated in 1940." Elsewhere in their report[1] the authors mention "SK mouse poliomyelitis," hence they presumably worked with the Columbia-SK virus which is a neurotropic agent immunologically indistinguishable from the encephalomyocarditis (EMC) and MM viruses.[2] The lack of effect of this drug on the course of the disease in mice experimentally infected with EMC and MM viruses is reported here. The phenosulfazole used in the present work was supplied by Dr. H. R. Cox of the Lederle Laboratories.

In Exp. 1, groups of 30 Swiss mice (Bagg strain) weighing 12 g each were infected with varying amounts of EMC virus intraperitoneally and treated with phenosulfazole by the same route. The drug was diluted to contain 6.25 mg/cc and given at 8 and 11 a. m. and 2 and 5 p. m. in doses of 0.5, 0.25, 0.25 and 1.0 cc respectively. Treatment was begun 17 hours after infection and continued for seven days with a total daily dose of 12.5 mg. Control mice were injected in the same manner as the treated group except that normal saline was used. Following infection the mice were observed for 17 days when the death ratios were calculated. It should be noted that few deaths occurred subsequent to the ninth day after infection. Selected animals in the treated and control groups were autopsied and the virus recovered by passage of brain tissue into normal mice.

In Exp. 2, groups of 16 mice weighing about 20 g each were infected and treated in a manner similar to that employed in Exp. 1, except that MM virus was used and the schedule of therapy was varied somewhat. Therapy was initiated in the 3 groups of treated mice as follows: (a) 2 hours before infection (b) 2 hours after infection and (c) 19 hours after infection. In each instance the initial dose was 8.33 mg followed by a course of treatment similar to that described above to give 16.7 mg daily. Because of evidence of toxicity the daily dosage was reduced to 12.5 mg per mouse on the second day and was maintained at this level until stopped 5 days after infection. It should be noted that in this experiment the titer of the MM virus in the control mice was at least $10^{-7.9}$. That the upper limit of infectivity could not have been much beyond this range is indicated by the

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[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Rep. Biol. and Med.*, 1948, **6**, 385.

[2] Warren, J., and Smadel, J. E., *Fed. Proc.*, 1948, **7**, 311.

TABLE I.
Phenosulfazole in the Treatment of Mice Infected with EMC or with MM Viruses.

Virus	Therapy: Initial dose (hr)	Therapy: mg/day	Dilution of infectious material: $10^{-5.0}$	$10^{-6.0}$	$10^{-7.0}$	$10^{-8.0}$	MLD 50%
EMC	0 (control)		30/30*	21/29	17/30	5/30	$10^{-7.0}$
	17 post infection	12.5	30/30	29/30	23/30	—	$10^{-7.3}$†
MM	0 (control)		16/16	12/16	12/16	11/16	$10^{-7.9}$†
	2 pre inf.	12.5‡	13/14	14/15	11/14	—	$10^{-7.3}$†
	2 post inf.	12.5‡	14/14	14/16	10/14	—	$10^{-7.2}$†
	19 post inf.	12.5‡	16/16	15/16	9/16	—	$10^{-7.1}$†

* Numerator = No. of mice dying; denominator = No. of mice in group.

† These figures represent minimal values. The exact titers are at least this great.

‡ Initial dose 8.33 mg/mouse. These mice received 16.7 mg drug per day during the first 24 hours and 12.5 mg per day subsequently. See text.

fact that this same preparation of frozen stored virus when titrated five days later had a titer of $10^{-7.5}$.

The results of the two experiments are summarized in Table I. These data indicate that treatment with phenosulfazole produced no reduction in mortality among the mice infected with either EMC or MM viruses. Furthermore, there was no indication that it delayed the onset or duration of the disease.

16983 P

Evaluation of the Effect of Darvisul upon Infection with SK Strain of Virus in Mice.*

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Darvisul, N-(2-thiazolyl)-phenol sulfonamide, or phenosulfazole was reported by Sanders, SubbaRow, and Alexander[1] to be effective against as many as 100 LD_{50} of a mouse virus, SK, when administration was begun 24 hours after intraperitoneal infection.

Through the courtesy of Dr. Sanders the virus was received in the form of two infected mouse brains in glycerine. The drug was received in ampules of 25% solution of lot No. 7-8522, through the kindness of Dr. Sanders and Dr. Leo Rane of the Lederle Laboratories. The plan of testing the drug was that outlined by Dr. Sanders and was rigidly observed.

The original brain material was made into a 10% suspension in 10% horse serum physiological salt solution. A titration was done by intraperitoneal inoculations of groups of 5 mice and found to be approximately 2 × 10^{-7}. A pool was made from the brains of mice sacrificed when moribund and the 10% suspension of this material served as the material with which the therapeutic effects of the compound were tested.

Albino mice of 20 g weight of a single strain were used. As shown in the table 50 mice were inoculated intraperitoneally with the respective concentrations of virus and 24 hours later administration of the chemical was begun by the same route. The stock solutions of compound were diluted 1:31 in physiological salt solution so that each ml contained 8 mg. The material was given

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Rep. Biol. and Med.*, 1948, 6, 385.

TABLE I.
Evaluation of Effect of Darvisul on SK Virus in Mice.

Dilution of virus	Treated No. survivors 5th day	Treated No. survivors 14th day	Controls No. survivors 5th day	Controls No. survivors 14th day
2×10^{-4}	0/25	0/25	0/25	0/25
2×10^{-5}	1/25	0/25	1/25	0/25
2×10^{-6}	7/25	2/25	7/25	2/25
2×10^{-7}	21/25	18/25	23/25	18/25

Date of Experiment = October 3, 1948.
Numerator = survivors. Denominator = total number inoculated.

intraperitoneally to 25 mice in amounts of 0.5 ml at 9 a.m., 0.25 ml at 1 p.m., 0.25 ml at 4 p.m., and 1.0 ml at 7 p.m. so that the total daily dosage was 16 mg. Control mice, 25 in number, received equivalent volumes of salt solution intraperitoneally.

The results are presented in Table I. It is apparent that no difference between treated and control mice was observed. It is concluded, therefore, that under the conditions of the experiment Darvisul had no therapeutic effect upon the infection of mice with the SK strain of murine encephalomyelitis virus.

16984

Behavior of the Thyroid toward Elements of the Seventh Periodic Group. I. Halogens and Thiocyanates.*

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It will perhaps surprise many readers of this report to learn that the thyroid will filter from the blood not only iodine but the other halogens as well. Further, while it is generally known that feeding thiocyanates to man and other animals will cause the development of goiter, thyroid hyperplasia and goiter have also been produced by feeding relatively large amounts of any of the halogens other than iodine. The evidence for these statements will be assembled, much of it from the literature, and an hypothesis offered that attempts to show that bromides, chlorides, fluorides, as well as thiocyanates all bring about thyroid enlargement by the same means, and to explain why the responses of the thyroid to all of these substances are similar.

Filtration of Halogens and Thiocyanates by the Thyroid. The first step in formation of thyroxine consists in filtering iodide from the blood. This filtration by the thyroid is remarkably effective, its concentrating power being of the order of 10,000. In an early experiment of Marine and Feiss[1] nearly 90% of a dose of 5 mg of KI was found in the goitrous thyroid of a dog in 10 minutes. In many more recent experiments with radioactive iodine similar results have been noted. Even when the synthesis of thyroxine is prevented by administration of thiourea and related substances, the thyroid can still filter iodine from the blood, first shown by Baumann, Metzger, and Marine[2] and subsequently by others. Selective filtration by the thyroid is not limited to iodine; in fact the gland behaves in a similar fashion with all the elements of the 7th group of the periodic table that have been studied, *viz.*, Cl, Br. Mn, and At, as well as with thiocyanates. It may be predicted that fluorine, rhenium, and technetium (element 43) will be found to behave in the same way.

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* A report of this work was made before the American Society of Biological Chemists at Chicago, May 14, 1947, *Fed. Proc.*, 1947, 6, 237.

[1] Marine, D., and Feiss, H. O., *J. Pharm. Exp. Therap.*, 1915, 7, 557.

[2] Baumann, E. J., Metzger, N., and Marine, D., *Endocrinol.*, 1944, 34, 44.

Baumann, Sprinson, and Marine[3] found more Br in rabbit thyroids than in other tissues, especially when the gland was hyperplastic, an observation that had been made by many other investigators. Ray and Desach,[4] after injecting subcutaneously small amounts of $MnCl_2$ daily for 6 days, noted an accumulation of Mn in all tissues, but they found that the thyroid had a concentration 2 to 10 times as great as any other tissue studied. And somewhat similar experiments were made with Astatine by Hamilton and Soley[5] using the tracer technic. This element, prepared by α particle bombardment of Bi by Corson, Mackenzie, and Segrè[6] is radioactive. It was found to behave as a metal rather than like the halogens, *e.g.*, it is not precipitated by $AgNO_3$ from nitric acid solution while it is precipitated by H_2S from an acid solution. Hamilton and Soley fed tracer doses of this isotope to guinea pigs with normal or with hyperplastic thyroids. They found many times more Astatine in the thyroid than in any other tissue they examined.

Experimental. We compared the concentration of Cl^- and SCN^- in thyroid and other tissues of albino rats. Both normal and goitrous animals were used. The goitrous rats were fed our stock food mixture to which 1% thiouracil[†] was added for 2 to 5 weeks. The tissues of two or more rats were pooled and treated as follows:

Finely minced tissues were analyzed for Cl^- by digestion over a free flame with 5 ml standard $AgNO_3$ and a few drops $KMnO_4$ if necessary (Eisenman[7]). For thiocyanate determination the finely minced tissues were weighed into 25 or 50 ml Erlenmeyer flasks and digested with 10-20 ml 4% alcoholic KOH in a boiling water bath. To limit evaporation a small funnel covered with a watch glass was placed in each flask while heating. After half an hour, the funnel and cover glass were removed, the contents of the flask evaporated to dryness, neutralized with 5 N H_2SO_4, adding 0.2 ml in excess and finally 1.5 ml 10% Na_2WO_4. After the precipitated protein had flocked out, the mixture was made up to 10 ml, centrifuged and filtered. A 75-80% aliquot was analyzed by the method of Baumann, Sprinson, and Metzger.[8]

Adult rats were used to measure the Cl^- content of tissues. Our stock diet consisting of mixed cereals, dried milk, yeast, meat scrap, and salts contains about 1.3% NaCl. Due to the special role of chlorides in the physiology of tissue fluids, one would hardly expect to find as much Cl^- in thyroid as in blood. However, thyroid did contain 2 or 3 times as much Cl^- as muscle while the Cl^- concentration of tissues having much blood, such as liver, was only slightly less than in thyroid. Some of the data of these tests are given in Table I.

In the thyiocyanate experiments adult rats were injected intraperitoneally with 2 ml of a 5% NaSCN solution and sacrificed by chloroform anesthesia 4 to 5 hours later. Blood was taken from the heart, the tissues dissected and prepared for analysis. Some of these data are given in Table II. A similar experiment was made on a 2.5 kg rabbit in which 7 ml of 5% NaSCN were injected intraperitoneally. This proved to be irritating, the rabbit showing signs of discomfort for about 15 minutes, but no hyperemia of the peritoneum was found at autopsy 5 hours later.

It will be seen from Table II that thyroid tissue always contained a greater concentration of SCN^- than any other tissue, though

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[3] Baumann, E. J., Sprinson, D. B., and Marine, D., *Endocrinol.*, 1941, **28**, 793.

[4] Ray, T. W., and Desach, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 228.

[5] Hamilton, J. G., and Soley, M., *Proc. Nat. Acad. Sci.*, 1940, **26**, 483.

[6] Corson, D. R., MacKenzie, K. R., and Segrè, E., *Phys. Rev.*, 1940, **57**, 459.

[†] We thank the American Cyanamid Company, who supplied the thiouracil.

[7] Eisenman, A. J., *J. Biol. Chem.*, 1929, **82**, 411.

[8] Baumann, E. J., Sprinson, D. B., and Metzger, N., *J. Biol. Chem.*, 1934, **105**, 269.

TABLE I.
Comparison of Cl^- Concentration in Thyroid and in Other Fresh Tissues.

No. rats	Treatment	Thyroid Cl, mg per g	Muscle Cl, mg per g	Pancreas Cl, mg per g	Liver Cl, mg per g
3	Normal	1.48	.50		1.26
5	”	1.19	.47		1.11
5	”	1.30	.50	1.20	1.07
5	”	1.27	.49	1.05	1.12
7	”	1.29	.51	1.00	1.13
4	”	0.75	.44		
2	Thiouracil	0.81	.43		
2	”	1.30	.47		1.03
2	”	1.32	.46		0.98
2	”	1.99*	.44	1.30	1.12
2	”	1.56*	.95		1.27

* These rats had snuffles-pneumonia.

TABLE II.
Comparison of Concentration of SCN^- in Thyroid and in Other Fresh Tissues.

No. animals	Thyroid, wt, g	Thyroid SCN, mg per g	Muscle SCN, mg per g	Liver SCN, mg per g	Blood SCN, mg per g	Kidney SCN, mg per g
2 rats	.209	.49	.21	—	.30	.43
2 ”	.147	.64	.18	—	.31	—
2 ”	.119	.71	.48	.42	.53	.66
3 ”	.259	.73	.51	.38	.40	.62
3 ”	.213	.70	.37	.40	.58	.66
2 ”	.125	.80	.60	.50	.54	.74
Rabbit	.179	.59	.30	.29	.26	.37

the kidney which is the excretory organ for thiocyanate had only a slightly lower concentration than the thyroid. The level of tissue SCN^- was largely determined by the elapsed time between injection and death.

The daily intake of iodine necessary to maintain a normal thyroid in an albino rat is only 2 or 3 μg. If 50 to 250 mg of halides are added daily to the food of rats, a not very excessive amount, they would receive 16,000 to 80,000 times as much halide as iodide. And since all halogens are selectively filtered by the thyroid one could reasonably expect the added halide to have a mass action-like effect on the iodine normally ingested, thus preventing it from reaching the thyroid. Such is the case. Experiments have been made with fluoride, chloride, bromide, and thiocyanate (which behaves like the halogens) and with each of these substances, thyroid hyperplasia was produced.

Thus Goldemberg[9] found that rats given NaF for several months develop very hyperplastic thyroids, as much as 8 times the normal size. Hibbert[10] produced thyroid hyperplasia in rats on low iodine intake by giving 2% $CaCl_2$ or 3% NaCl. These observations were confirmed and extended by Remington[11] who fed diets containing 1 to 4% of NaCl. The thyroid iodine was reduced to one-third of the amount in the control animals. Morruzzi[12] and Simon[13] found on continued feeding of NaBr to young rats that thyroid I was decreased and goiter developed. The many more recent experiments in which goiter has been produced by giving thiocyanates to man and other animals are well known and need not be specifically cited.

Our observations and those from the literature are summarized in Table III. Table III indicates (1) that the thyroid selectively

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[9] Goldemberg, L., *C. R. soc. biol.*, 1926, **95**, 1169.

[10] Hibbert, J. S., *Arch. Surg.*, 1933, **26**, 648.

[11] Remington, R. E., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 652.

[12] Morruzzi, G., *Boll. soc. ital. biol. sper.*, 1940, **15**, 733.

[13] Simon, I., *Boll. soc. ital. sper.*, 1941, **16**, 562.

TABLE III.
Summary of Thyroid Response to Administration of Group VII Salts.

	Selectively filtered by thyroid	Reduces thyroid iodine	Produces thyroid hyperplasia and goiter
F	?	?	+
Cl	+	+	+
Br	+	+	+
I	+	—	—
SCN	+	+	+
Mn	+	?	?
At	+	?	?

filters from the blood SCN^-, the halides, and other members of group VII of the periodic table. This inference is based on the greater concentration of these ions found in the thyroid compared with other tissues; (2) that the reduction of thyroid iodine is brought about by giving 0.1 to 0.4 g SCN^-, F^-, Cl^-, Br^- to rats daily for several weeks (or greater doses to larger animals); and (3) that the development of thyroid hyperplasia follows as a consequence of the lowering of thyroid iodine.

Discussion. The investigations mentioned above as well as others have all been concerned with but one of these ions. There has been no very satisfactory explanation offered of how any of them act. In fact most workers have been content to go no farther than to present their findings. For example Wolff, Chaikoff, Taurog, and Rubin[14] in studies on thiocyanate goiter concluded that SCN^- inhibited the uptake of I by the thyroid, but no explanation of the inhibition was offered. In a study of the uptake and discharge of I by propylthiouracil and thiocyanate-induced goiters, VanderLaan and Bissell[15] found that SCN^- did interfere with the uptake of I but this could be prevented by giving large amounts of I simultaneously. Propylthiouracil, on the other hand, inhibited thyroid hormone synthesis but did not interfere with the "trapping" mechanism, an effect similar to that described by Baumann, Metzger, and Marine[2] for thiourea a number of years before. This study was extended by J. E. and W. P. VanderLaan[16] who found that SCN^- inhibited the preferential accumulation of small amounts (<1.0 mg) of iodide and that this inhibition persisted for 5 hours. Furthermore they found that bromides in a dose 500 times greater than that of the injected iodide will not cause significant I displacement from the thyroid. In this experiment apparently a single dose of bromide was used though no details are given. It does not necessarily conflict with those of Morruzzi[12] and of Simon,[13] for the latter fed bromides continuously for months and uniformly found thyroid iodine greatly diminished under these conditions.

In all the thyroid-halogen-SCN^- work so far considered, massive doses of halide or thiocyanate have almost always been used, many hundreds or thousands of times as much as the I ingested. We believe that these anions exert a mass action-like effect; as a consequence inorganic iodide is washed out of the thyroid and new supplies of I are prevented from being taken up. The iodine present as thyroxine and diiodotyrosine is used up in the course of a few weeks, and since new supplies of I cannot be received, thyroid hyperplasia and goiter develop as soon as the thyroid I falls below a critical level. Not only do the halides and SCN^- behave this way toward I, but we[3] have found that large doses of chlorides remove bromides from the thyroid. We believe that continued administration of relatively large amounts of any halide will wash out any other halide present in small amount.

Thiocyanates behave in the same way as do the halogens. For they too accumulated preferentially in the thyroid and at the same time washed iodide out, thus producing thyroid hyperplasia. This similar behavior of SCN^- is not surprising; rather it is to be expected from the position of SCN^- at the end of the Hofmeister anion series (acetate$>$ $Cl^- > Br^- > I^- > SCN^-$, etc.) Since the effects of these anions on protein swelling, surface tension, and on many other phenomena increase as the SCN^- end of the series is approached, it is reasonable to suppose that the ability of the thyroid to filter these ions from the blood would be affected similarly.

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[14] Wolff, J., Chaikoff, I. L., Taurog, A., and Rubin, L., *Endocrinol.*, 1946, **39**, 140.

[15] VanderLaan, W. P., and Bissell, A., *Endocrinol.*, 1946, **39**, 157.

[16] VanderLaan, J. E., and VanderLaan, W. P., *Endocrinol.*, 1947, **40**, 403.

A question that arises is how the thyroid differentiates between the halogens and other ions. A point of similarity is found in the arrangement of their electrons. All have 7 in their outer shells consisting of 2 in the s and 5 in the p groups. We suggest that this characteristic electron arrangement of the halogens is one that may enable thyroid tissue to effect this differentiation. At the same time it would explain why *all* halides are filtered from the circulating medium in preference to other ions. While thiocyanates closely resemble the halides in their chemical properties, and while the behavior of tissues towards halides and thiocyanates is similar we do not know whether their nuclear structure is correspondingly alike.

Further to support this view one should note the preferential accumulation of Mn and At by the thyroid. Like the halogens these elements of the seventh periodic group also have 7 electrons in their outer shells but instead of 2 s and 5 p electrons, the arrangement is believed to be reversed. And as stated in the first part of this paper one would also expect the remaining elements of group VII, *viz.*, technetium (element 43), and rhenium, to be preferentially filtered from the blood.

It becomes a matter of great interest to determine the relative avidity of the thyroid for these elements, to determine whether the preference follows a definite order, and to note the effect of the reversal of the electrons in the outer shell.

Summary. 1. Thyroid filters preferentially and accumulates thiocyanates as well as all the elements of the seventh periodic group that have been studied, *viz.*, Cl, Br, I, Mn, At.

2. All halides and thiocyanates when administered in relatively large amounts compared with iodine wash inorganic iodide from the body and prevent new supplies of iodine from being received by the thyroid, thus producing thyroid hyperplasia and goiter. It is suggested that these ions exert, at least in part, a mass action-like effect.

3. It is further suggested that similarity in arrangement of electrons in the outer shell of the elements of the seventh periodic group enables the thyroid to differentiate between them and other elements.

16985

Water Content of Rat Uterus and Oviducts During Proestrus and at End of Heat.*

D. LOUISE ODOR AND RICHARD J. BLANDAU. (Introduced by C. E. Tobin.)

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During the period of sexual receptivity in the rat the uterine cornua are distended with fluid which is retained by the tonic contractions of the cervix.[1,2] The appearance of the fluid within the lumina coincides with a significant decrease in uterine tissue water.[3] Also during heat several of the ampullar loops of the oviducts become greatly distended with

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* This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

[1] Long, J. A., and Evans, H. M., *Mem. Univ. Calif.*, 1922, **6**, 1.

[2] Blandau, R. J., *Am. J. Anat.*, 1945, **77**, 253.

[3] Astwood, E. B., *Am. J. Physiol.*, 1939, **126**, 162.

fluid and serve as the reservoir for the newly ovulated ova.[4,5] We were interested in determining whether changes in the tissue water of the oviducts paralleled those observed in the cornua and whether such changes could explain the source of the luminal fluid dilating the loops of ampullae.

Materials and Methods. Forty-one sexually mature female rats, of Wistar strain, weighing 148 to 205 g, were carefully observed to determine the onset of sexual receptivity.[6] Twenty of these females were killed at the end of heat or at the time of greatest loss of uterine tissue water.[3] The remaining 21 females were killed during proestrus or when the tissue water had reached its maximum.[3] The cornua, oviducts, and ovaries were removed intact. The utero-tubal junctions were cut and the oviducts and ovaries were put into Ringer's solution. The mesometria were carefully trimmed from the cornua and the vagina from the cervix. Each horn was then split open along its entire length, blotted on absorbent paper and weighed in weighing bottles. With the aid of iridectomy scissors and a binocular dissecting microscope, the oviducts were trimmed free of ovaries, periovarial sacs and all excess adipose tissue. A fine pipette was inserted into the fimbriated end of each oviduct and a stream of air blown through it to remove the luminal fluid. With the pipette still in place, the oviduct was removed from the Ringer's solution, brought onto absorbent paper and blotted, and the flow of air continued until it was free of fluid. The oviducts were placed in weighing dishes and weighed on a micro-analytical balance. The cornua and oviducts were subsequently dried in an oven at 110°C and reweighed. The loss of water in the cornua and oviducts was expressed in terms of per cent per 100 grams of body weight.

Results. The average percentage of water in the tissues of the cornua of females killed during proestrus was 81.8 ± 1.05 and at the end of heat 80.5 ± 0.48 (Tables I and II). The average percentage of tissue water in the oviducts in females killed during the period of proestrus was 76.5 ± 1.25 and at the end of heat 75.7 ± 0.84 (Tables I and II). There is a significant decrease in the uterine tissue water at the end of heat when compared with that of uteri removed during proestrus. The average figures presented in the tables correspond closely with those published earlier by Astwood.[3] In using the vaginal smear technic for timing his animals he reported the average per cent of uterine water during proestrus as 82.77 ± 0.35 and during late estrus as 80.37 ± 0.17.

Although there is a slight decrease (0.8%) in the tissue water of oviducts examined at the end of heat when compared with those removed during proestrus this decrease does not follow the pattern observed in the cornua. The significance of the difference between the percentage of water of the oviducts removed during proestrus as compared to those removed at the end of heat was tested by the Student's "t"-technic. The "p" value obtained ("p" = 0.027) indicates that there is no statistical significance in the differences of the two groups.

Discussion. The significant decrease in uterine tissue water between the period of proestrus and the end of heat has been confirmed. This increase in the water content of the cornua has been previously demonstrated to be due to the specific action of estrogens.[7,8] The sudden decrease in uterine tissue water during heat is thought to be due either to a decrease in estrogen production or to the action of progesterone which has an inhibitory influence on the deposition of tissue water. The origin of the fluid which dilates the ampullae of the oviducts during heat is probably not from the tissue water of this organ. The possibility of retrograde flow of uterine fluid in the normal female has been suggested but ligation of the oviducts just above the utero-tubal junction does not influence the extent to which the dilatation progresses. In addition, dyes

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[4] Huber, C. G., *J. Morph.*, 1915, **26**, 247.

[5] Odor, D. L., and Blandau, R. J., *Anat. Rec.*, 1947, **97**, 84.

[6] Blandau, R. J., Boling, J. L., and Young, W. C., *Anat. Rec.*, 1941, **79**, 453.

[7] Astwood, E. B., *Endocrin.*, 1938, **23**, 25.

[8] Van Dyke, H. B., and Ch'en, G., *Am. J. Anat.*, 1936, **58**, 473.

TABLE I.
Total Weight, Dry Weight, and Percentage Water of Uteri and Oviducts of Rats Killed at the End of Heat.

			Uteri			Oviducts		
Animal No.	Hr after onset of heat	Body wt	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water
4	15	152	256.1	51.6	79.8	9.212	2.279	75.3
5	17	165	349.0	66.1	81.1	10.317	2.492	75.9
7	14	180	216.2	40.8	81.1	8.069	1.939	76.0
15	13	195	228.7	42.9	81.2	7.617	1.899	75.1
17	12	185	240.8	48.0	79.7	7.422	1.775	76.2
18	12	180	296.2	57.3	80.7	9.128	2.168	76.3
19	12	185	289.1	56.9	80.3	8.178	1.937	76.3
20	12	148	438.8	84.9	80.7	10.475	2.528	75.9
21	14	149	254.6	51.5	79.8	9.351	2.168	76.8
22	14	175	222.5	45.3	79.7	7.166	1.858	74.1
23	12	172	259.7	50.2	80.7	7.014	1.641	76.6
24	12	150	230.9	45.2	80.4	7.608	1.800	76.4
25	12	200	298.0	57.9	80.1	5.874	1.419	75.8
26	13	170	288.7	56.1	80.6	6.589	1.558	76.7
27	14	155	230.3	45.7	80.2	8.049	1.937	75.9
28	12	175	346.4	67.0	80.7	8.502	2.041	76.0
29	12	152	254.4	50.4	80.2	8.564	2.068	75.9
30	13	164	257.6	50.1	80.5	6.358	1.560	75.5
31	13	172	262.6	51.4	80.4	7.561	1.958	74.5
32	13	182	411.3	76.9	81.3	6.875	1.827	73.5
	Avg		281.6	54.8	80.5	7.996	1.943	75.7
	σ		59.91	11.04	0.48	1.217	0.284	0.84

TABLE II.
Total Weight, Dry Weight and Percentage Water of Uteri and Oviducts of Rats Killed During Proestrus.

			Uteri			Oviducts		
Animal No.	Hr after onset of heat	Body wt	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water
8	88	180	300.2	51.0	83.0	8.420	2.052	75.6
9	88	170	281.6	86.0	82.0	7.829	1.966	74.9
10	89	165	361.5	62.4	82.7	9.382	2.257	76.0
11	89	205	285.9	49.6	82.7	6.756	1.721	74.5
14	84	175	268.2	45.9	82.9	7.504	1.919	74.5
16	88	174	383.7	69.4	81.9	8.190	2.040	75.1
51	84	172	284.9	50.3	82.5	8.389	1.863	77.8
52	86	174	243.3	44.3	81.8	7.926	1.803	77.3
53	86	222	319.4	57.3	82.1	5.416	1.255	76.8
54	87	184	283.2	50.5	82.2	8.401	1.830	78.4
55	88	182	346.6	69.7	79.9	8.145	2.007	75.4
56	87	166	391.0	70.5	82.0	9.645	2.183	77.4
57	87	160	325.7	62.9	80.7	8.196	1.962	76.1
58	87	176	258.6	46.0	82.2	8.118	1.773	78.2
59	88	171	209.3	40.3	80.8	6.762	1.597	76.4
60	82	186	213.1	45.0	78.4	7.446	1.853	75.2
61	85	188	354.8	65.3	81.6	7.372	1.752	76.2
62	85	178	318.1	57.5	82.0	8.651	1.981	77.1
63	83	180	212.2	39.3	81.5	9.355	2.003	78.6
64	88	182	293.1	53.6	81.7	8.033	1.834	77.2
65	88	180	280.2	49.4	82.4	8.092	1.789	77.9
	Avg		295.9	55.5	81.8	8.001	1.878	76.5
	σ		51.71	11.66	1.05	0.932	0.206	1.25

injected into the cornua during heat do not find their way into the ampullae of the oviducts. Follicular fluid may contribute to the luminal fluid but the ampullar loops have already become distended before ovulation occurs. It has been postulated that there is an increased flow of fluid from the coelomic cavity into the oviduct at the time of ovulation.[9,10] From the investigations of Alden,[11] and our unpublished data, it seems likely that the periovarial sac opening is functionally closed during the period of heat.

Summary. The water content of the uterus and oviducts of adult rats during the period of proestrus and the end of sexual receptivity has been determined. There is a significant decrease in the water content of the uterine tissue during the period of heat. No significant decrease was found in the water content of the oviducts examined during this same period.

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[9] Horst, C. J., *S. African J. M. Sc.*, 1943, **8**, 41.

[10] Westman, A., *Acta Obstet. et Gynecol. Scand.*, 1926, **5**, 1.

[11] Alden, R. H., *Anat. Rec.*, 1942, **83**, 421.

16986

Coproantibody Response in Humans Following T.A.B.* Vaccination.

R. J. GOODLOW, S. C. RITTENBERG, AND J. H. SILLIKER.
(Introduced by John F. Kessel.)

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The value of coproantibody determination in the laboratory diagnosis of various enteric infections has been discussed recently by Harrison and Banvard.[1] The presence of agglutinins in feces was first described by Davies,[2] but this work was overlooked until the Russian investigators, Predpechensky and Moroz,[3] Skochko,[4] and Yampolsky, Gorfunkel and Aronina,[5] utilized the presence of fecal antibodies in the laboratory diagnosis of bacillary dysentery.

According to all previous investigators, agglutinins in the feces appear sooner than agglutinins in the blood serum in cases of actual enteric infection. This might suggest a local formation of the antibody in the tissue of the intestinal tract. Burrows and coworkers[6,7] indicate that antibody is excreted in the feces of animals and humans vaccinated with *V. cholerae* antigen. It is difficult to conceive how the subcutaneous injection of dead cells could stimulate the local formation of antibodies in the intestinal tract. Their appearance under these circumstances might indicate rather some mechanism of spill over from the blood stream. This view is substantiated in part by the observations of the Russian workers who found a high correlation between the presence of cellular exudate in the stool and fecal antibodies in cases of bacillary dysentery. Stools which contained large numbers of red blood cells and leucocytes commonly contained agglutinin in higher titer than stools which showed little or no cellular exudate. Burrows *et al.*,[7] however, emphasize that fecal antibodies reach their peak titer before serum antibody and tend to decline before the maximum serum

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* T.A.B.—Typhoid, Paratyphoid A and Paratyphoid B vaccine.

[1] Harrison, P. E., and Banvard, J., *Science*, 1947, **106**, 188.

[2] Davies, A., *Lancet*, 1922, **2**, 1009.

[3] Predpechensky, S., and Moroz, O., *Zh. Mikrobiol. Epidemiol.*, 1940, **7**, 3.

[4] Skochko, T. I., *Zh. Mikrobiol. Epidemiol. Immunol.*, 1942, **3**, 59.

[5] Yampolsky, S. M., Gorfunkel, D. M., and Aronina, V. B., *Pediatriya*, 1944, **5**, 13.

[6] Burrows, W., Havens, I., *J. Inf. Dis.*, 1948, **82**, 231.

[7] Burrows, W., Elliott, M. E., and Havens, I. J., *J. Inf. Dis.*, 1947, **81**, 261.

TABLE I.

Blood Serum Agglutinin Titer and Coproantibody Response in Humans Following T.A.B. Vaccination.

		Serum titer† Day				Stool titer. 0 except where indicated Day									
Subject	Organism*	0	7th	14th	24th	0	3	5	7	10	12	14	17	19	24
1	T	0	640	1280										§	
	A	0	80	640										”	
	B	0	160	320										”	
2	T	0	320	1280	1280					§				”	
	A	0	160	640	1280					”				”	
	B	0	20	320	640					”				”	
3‡	T	0	0	80	320							§			
	A	0	80	640	320							”			
	B	0	80	1280	320							”			
4	T	20	320	320	640									8	
	A	0	80	640	640									8	
	B	0	40	80	640	5									
5‡	T	0	640	1280	160										
	A	0	160	320	320									16	
	B	0	20	160	160										
6	T	0	160	1280	1280										
	A	0	20	1280	2560										16
	B	0	80	640	2560										
7	T	0	320	640	640										
	A	0	120	320	1280										
	B	0	20	160	640										
8	T	0	160		1280										
	A	0	160		1280										
	B	0	20		640										32
9	T	20	160	80	1280		8								
	A	80	160	1280	1280									32	
	B	0	20	80	640										
10	T	40	640	640	640										
	A	0	80	640	1280										32
	B	0	40	640	640										
11	T	0	160	640	640			8							
	A	0	20	1280	640			8						32	
	B	0	0	160	640			8							
12	T	0	320	1280	2560										
	A	0	40	640	640										
	B	0	20	160	160										
13	T	40	640	1280	640										§
	A	0	80	1280	640								§	16	”
	B	20	80	1280	1280								”		”
14	T	80	160	1280	1280										
	A	0	40	1280	2560										
	B	0	20	1280	2560										
15	T	40	640	1280	640								8		
	A	0	80	1280	640									64	
	B	10	80	640	1280				8						
16‡	T	0	40	320	160		§	§			16		§		
	A	0	0	160	160		”	”			16		”	64	
	B	10	0	80	0		”	”					”		
17	T	10	320	640	160						§				
	A	0	20	160	320						”				
	B	0	20	320	160						”				
18	T	160	640		1280									8	
	A	0	20		1280									64	32
	B	0	20		320									16	
19	T	80	640	1280	1280									64	
	A	0	20	1280	2560									64	
	B	0	20	640	1280										
20	T	40	640	1280	2560					32	32				
	A	10	320	1280	1280					8	64			64	
	B	0	80	1280	640					16	16				

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* T—titer vs. *S. typhi*; A—titer vs. *S. paratyphi* A; B—titer vs. *S. paratyphi* B.

† Expressed as reciprocal of highest positive dilution.

‡ No history of previous T. A. B. vaccination. § No record.

titer is reached. They conclude that fecal antibody is independent of serum antibody and therefore not derived from it.

Since many of our students are routinely vaccinated with T.A.B. vaccine, it appeared to us an ideal opportunity to study the relation of vaccination and coproantibody excretion with antigens different from the cholera antigen used by Burrows.

Experimental. Twenty human volunteers were chosen for vaccination. Of this group, 7 were females and 13 males. Three of the subjects had no previous history of T.A.B. vaccination. The remainder had had no injections within the previous 2 years.

The vaccine contained 1,000 million *S. typhi* per ml, 500 million *S. paratyphi A* per ml, and 500 million *S. paratyphi B* per ml. The initial injection consisted of 0.5 ml of vaccine administered subcutaneously, followed by 1.0 ml injections, similarly administered, 7 and 14 days later.

Agglutination tests against the homologous organisms were performed using blood serum and fecal extract collected before the initial injection of vaccine, periodically throughout the series of injections and for 10 days after the final injection. The agglutination titers were determined by the usual tube technic.

The stool extracts were prepared as follows using the technic described by Harrison.[8] Morning stool specimens were collected in clean stoppered bottles the day of the test. The samples for the most part were formed or semi-formed. To each bottle approximately 2 volumes of 0.6% formalinized saline were added per volume of feces. The mixture was then homogenized in a mechanical shaker for 30 minutes and placed in the refrigerator for overnight extraction. The following morning approximately 10 ml of the mixture was placed in a centrifuge tube and spun at 3000 r.p.m. for one hour. Five ml of the relatively clear supernate was further diluted with an equal volume of formalinized saline and recentrifuged for the same period of time. In most cases this procedure gave a clear solution which was considered a 1:4 dilution of feces. In certain instances the extract did not clear completely, but this did not appear to affect materially the subsequent results. It seems to us, however, that should the determination of coproantibodies come into general use, better and more rapid methods of clarification of the stool extract must be developed perhaps by the use of settling agents. The fecal extracts were used as a source of agglutinin in the usual tube agglutination test technic. The agglutination tubes were incubated at a temperature of 45°C overnight and read with the aid of the concave surface of a microscope mirror as is done commonly in the reading of Kahn tests.

The data obtained are presented in Table I. With one exception (No. 16), all subjects developed significant blood serum agglutinin titers against all 3 organisms. In most cases the maximum titer was reached on the fourteenth day following the initial injection of vaccine. No agglutinins were detected in fecal extracts of 7 subjects at any time during the course of the experiment despite the presence of significant serum titers. Of the remaining subjects fecal agglutinins against only one organism in the vaccine mixture were found in 5 individuals, against 2 organisms in 3 subjects and against all 3 organisms in 5 subjects. Seven volunteers showed fecal agglutinins against *S. typhi,* 14 against *S. paratyphi A*, and 5 against *S. paratyphi B* at some time during the course of the experiment. The appearance of fecal agglutinins against the homologous organisms in a particular individual was extremely irregular, in 18 instances being detected only once, in 6 instances twice, and in 2 cases 3 times. No correlation is apparent between the presence of fecal antibody and the antibody titer of the blood serum; nor is there any obvious relationship between fecal antibody appearance and the time and number of antigen injections.

Summary. These results fail to indicate any rise in fecal agglutinin titer prior to the appearance of blood serum agglutinins. In this respect and also in regard to the irregularity of antibody appearance in the feces of human subjects following T. A. B. vaccination our results differ from the regular and rapid appearance of coproantibody reported to follow cholera vaccination.

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[8] Harrison, P., personal communication.

16987

Effect of Zinc on Serum Alkaline Phosphatase Activity in Patients With and Without Cancer.*

OSCAR BODANSKY AND OLGA BLUMENFELD.

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Roche and his associates[1,2] have reported that a concentration of 10^{-5} M Zn^{++} activated the serum alkaline phosphatase activity in 96 out of 101 individuals who were either normal or had non-malignant disease; the degree of activation ranged from 0 to 87%. In contrast, this concentration of Zn^{++} inhibited the serum alkaline phosphatase activity in 156 out of 185 patients with cancer; the effects in this group ranged from an activation of 24% to an inhibition of 73%.

Upon subjecting these results to statistical analysis, we found that the mean values were 25% for the degree of activation and 14% for the degree of inhibition. The difference between these two values was 39% or about 20-fold the standard error of the difference between the two means (2.1%) and hence highly significant. It was, therefore, considered of importance to determine the extent to which Roche's results were applicable to a population of patients with cancer in this hospital and, in addition, to investigate the effects of other concentrations of Zn^{++}.

The method for the determination of serum alkaline phosphatase was essentially that of A. Bodansky.[3] The pH, determined electrometrically, of the serum-substrate-buffer mixture in the absence of any added Zn^{++} was 8.75 ± 0.05. It was found, in preliminary experiments, that addition of $ZnSO_4$ to give the desired concentrations of Zn^{++} caused a shift of the pH towards the acid side. Consequently, in all determinations where Zn^{++} was present, appropriate adjustment to give a final pH of 8.75 was made by the addition of a very small volume of 0.1 or 0.75 N NaOH. The pH was checked electrometrically in all determinations of phosphatase activity reported in the present work. The effect of Zn^{++} was evaluated as the ratio of the phosphatase activity in the presence of this ion to that in its absence.

Results. Table I shows that in a group of normal individuals and in patients without malignancy, the ratios at 10^{-5}, 10^{-4}, and 10^{-3} M Zn^{++} were, respectively, 1.01, 0.89, and 0.50. When these results were evaluated statistically, it was found that only the latter two values represented significant changes (Footnote, Table I). In other words, in this group, 10^{-5} M Zn^{++} had no effect on the serum alkaline phosphatase activity, whereas 10^{-4} M Zn^{++} inhibited the phosphatase activity about 10% and 10^{-3} M inhibited it about 50%.

The effect of Zn^{++} on the activity of serum alkaline phosphatase activity was studied in a group of patients with cancer in which the following were the types chiefly represented: leukemia, Hodgkin's disease, carcinoma of the colon, cervix, uterus, stomach, bladder, and breast. Examination of the ratios at each concentration of Zn^{++} showed that there was no apparent correlation of these ratios to age, sex, type of malignancy, level of phosphatase activity, or the pre-operative or post-operative state of the patient. Accordingly, the mean value for the effect of a particular concentration of Zn^{++} was calculated for the whole group of patients. The ratios at 10^{-5}, 10^{-4}, and 10^{-3} M Zn^{++} were, respectively, 1.00, 0.90, and 0.45. Again, only the latter two

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* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

[1] Roche, J., van Thoai, N., Marcelet, J., and Desruisseaux, G., *C. R. Soc. de biol.*, 1946, **140**, 632.

[2] Roche, J., van Thoai, N., Marcelet, J., Desruisseaux, G., and Durand, S., *Bull. Acad. Med., Paris*, 1946, **130**, 294.

[3] Bodansky, A., *J. Biol. Chem.*, 1933, **101**, 93.

TABLE I.
Effect of Zinc Ion on Serum Alkaline Phosphatase Activity in Patients With and Without Cancer.

Group	No. of cases	Ratio of phosphatase activity in presence of zinc to that in its absence		
		Range	Mean	Stand. dev.†
Individuals without cancer*				
a. At 10^{-5} M Zn^{++}	25	0.87-1.12	1.01	0.056
b. At 10^{-4} M ”	14	0.81-1.04	0.89	0.066
c. At 10^{-3} M ”	16	0.42-0.60	0.50	0.055
Patients with cancer				
d. At 10^{-5} M Zn^{++}	39	0.94-1.07	1.00	0.025
e. At 10^{-4} M ”	22	0.77-1.00	0.90	0.058
f. At 10^{-3} M ”	24	0.27-0.60	0.45	0.079

* This group consisted of normal individuals and patients with miscellaneous diseases, chiefly heart disease. We are indebted to Dr. Harry Gold for the use of the latter patients from the Beth Israel Hospital Cardiac Clinic.

† The values for the standard deviation were used in calculating "P" values. The "P" values for the comparison of groups *a* with *d*, *b* with *e*, and *c* with *f* were, respectively, 0.4, 0.6, and 0.04. The "P" values for the comparison of groups *a* with *b*, *a* with *c*, *d* with *e*, and *d* with *f* were all less than 0.01.

values represented significant changes. Thus, in patients with cancer, as in those without, 10^{-5} M Zn^{++} had no effect on the serum alkaline phosphatase activity, whereas 10^{-4} and 10^{-3} M Zn^{++} produced significant decreases.

Statistical treatment showed that there was no significant difference between the mean values for the ratios of the individuals without cancer and the patients with cancer at any of the 3 concentrations of Zn^{++}. In view of the high "P" values for these differences, it was considered very unlikely that the extension of the study to larger numbers of patients would bring the "P" value down to 0.01 and render the differences significant.

Summary. A concentration of 10^{-5} M Zn^{++} failed to affect the serum alkaline phosphatase activity in individuals either with or without cancer. These results are not in agreement with the findings of Roche *et al.*[1,2] that this concentration of Zn^{++} activates the serum alkaline phosphatase activity of individuals without cancer and inhibits this activity in patients with cancer. Concentrations of 10^{-4} and 10^{-3} M decreased the serum alkaline phosphatase activity, but the extent of these decreases was the same in patients with and without malignancy.

16988

Multiplication of Influenza Virus in Dead Chick Embryos.

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Recently, the cultivation of *Rickettsia prowazeki* in dead chick embryos was described by Rabinowitz, *et al.*[1] These workers showed that the rickettsiae employed multiplied abundantly in embryos which, although dead, still contained surviving cells. They confirmed the earlier work of Bucciante[2] and demonstrated that dead embryos may contain living cells for surprisingly long periods. After holding 3-day-old embryos at 4°C for 24 hours, then at room temperature for 7 days, and finally at 37°C for 16 days; they were able to culture living cells from the embryo or its membrane in plasma clots.

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[1] Rabinowitz, E., Aschner, M., and Grossowicz, N., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 469.

[2] Bucciante, L., *Arch. exp. Zellforsch.*, 1931, **11**, 397.

It seemed of both practical and theoretical interest to determine whether influenza virus would multiply in dead chick embryos. A number of experiments were carried out in which the time and temperature, at which embryos were held, were varied over wide ranges. The results obtained indicate that the PR8 strain of influenza A virus is capable of multiplication in the tissues of dead chick embryos if incubation at 35°C is sufficiently prolonged. They show also that the virus multiplies in dead embryos to an extent comparable to its multiplication in the living embryo.

Materials and Methods. The PR8 strain of influenza A virus was employed. It was cultivated in the allantoic sac of 9-day embryos which were incubated at 39°C before inoculation and at 35°C thereafter. Infected allantoic fluid gave hemagglutination and embryo infectivity titers of the order of 1:2000 and $10^{-8.5}$, respectively. Infected fluid was diluted in sterile nutrient broth; 0.2 cc of either a 10^{-3} or 10^{-6} dilution was employed as the inoculum.

The embryos of White Leghorn eggs were used. They were incubated at 39°C for periods ranging from 5-10 days. In certain experiments, after inoculation of the virus into the allantoic sac, embryos were held at room temperature for 3-10 days, and thereafter incubated at 35°C for 2-10 days. In other experiments, normal embryos were chilled at 4°C for either 20 or 96 hours, some of the former then held at room temperature for 3 days, after which they were inoculated into the allantoic sac with virus. Following this, the embryos were held at room temperature for an additional period of 2-10 days, and finally incubated at 35°C for 2-10 days. Groups of 2-3 embryos were employed and a variety of different experimental conditions were used. Specimens of allantoic fluid were removed repeatedly from individual embryos, usually at intervals of 2 days. In this way it was possible to follow the multiplication of the virus in individual embryos.

Measurement of the concentration of virus in the extra-embryonic fluid was carried out by the hemagglutination technique as described previously.[3] Washed chicken RBC in a final concentration of 0.25% were used. The allantoic fluid dilution-RBC mixtures were held at room temperature for one hour. The titration end point was taken as that dilution which gave a 2+ reaction. Hemagglutination-inhibition tests[3] with specific immune rabbit sera were carried out with a constant dilution of serum capable of inhibiting hemagglutination by 128 units of the homologous virus.

Virus infectivity titrations were carried out as described previously.[4] Serial tenfold dilutions of fluid were inoculated into the allantoic sac of 9-day chick embryos and, after incubation at 35°C for 48 hours, the allantoic fluids were removed and tested by the hemagglutination procedure. The infectivity end point was calculated in the usual way.

Results. It was found that the time required to kill embryos at room temperature varied somewhat with their age. Thus, 5-day embryos held at room temperature for one day were almost always dead; 8-day embryos held for 2 days were usually dead, and invariably when held for 4 days; 10-day embryos held for 3 days were usually dead, and invariably when held for 4 days. Only 5-day embryos were held at 4°C and in every instance it was found that 20 hours at this temperature resulted in death. Embryos were considered to be dead when spontaneous movements had ceased, blood vessels showed no pulsation and no further development occurred.

The results obtained with embryos held for long periods at room temperature after inoculation with the PR8 strain are shown in Table I. It was found that multiplication of the virus did not occur when embryos were kept at room temperature for periods up to 8 days and not incubated at 35°C thereafter. However, with inoculated embryos which had been held at room temperature for 7-10 days, and then incubated at 35°C for periods ranging from 2-10 days, definite evidence of mul-

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[3] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[4] Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

TABLE I.
Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at Room Temperature for Long Periods After Inoculation.

		After inoculation			Allantoic fluid	
Age of embryos (days)	Inoculum 0.2 cc intra-allantoic (dilution)	Room temp. 22-25°C (days)	35°C (days)	No. embryos	Hemagglut. titer*	Embryo-infectivity titer
5	10^{-3}	3-8	—	3	0,0,0	
7	"	"	—	"	0,0,0	
9	"	"	—	"	0,0,0	
5	"	7	2	2	8 16	
"	"	"	4	"	128,128	
"	"	"	8	"	256,256	$>10^{-5}$
8	"	"	2	"	0,0	
"	"	"	4	"	0,128	
"	"	"	8	"	512,2048	$>10^{-5}$
10	"	"	2	"	0.0	
"	"	"	4	"	0.0	
"	"	"	8	"	0 32	
5	"	10	3	"	0.32	
"	"	"	5	"	8.256	
"	"	"	7	"	512,2048	$>10^{-5}$
8	"	"	3	"	0 0	
"	"	"	5	"	0.32	
"	"	"	7	"	C,128	$>10^{-5}$
5	10^{-6}	7	2	3	0,0,128	
"	"	"	6	"	C,1024.1024	
"	"	"	10	"	C,2048,2048	
5	"	10	3-10	"	0,0.0	

* Hemagglutination titer of allantoic fluid from individual embryos.
C = Bacterial contamination.

tiplication of the virus was obtained. In contrast to what occurs in the allantoic sac of living embryos,[5] maximum hemagglutination titers were not obtained with dead embryos until the period of incubation at 35°C had been extended to 6 or 8 days. In certain instances hemagglutination titers as high as 1:2000 and embryo infectivity titers of at least 10^{-5} were obtained with fluid from dead embryos. Hemagglutination-inhibition tests with immune sera served to identify the virus; complete inhibition was demonstrated repeatedly with anti-PR8 serum, while anti-Lee serum did not inhibit the reaction. Even when the inoculum was as small as 10^{-6}, *i.e.* approximately 10^2 E.I.D., multiplication of the virus occurred. It is noteworthy that inoculated embryos which were held for 10 days at room temperature were capable of supporting the multiplication of influenza virus when they were incubated at 35°C. It should be reiterated that, irrespective of the age of the embryos employed, none was capable of surviving for longer than 4 days at room temperature.

The results obtained with embryos which were killed by chilling at 4°C, and held for various periods at room temperature before inoculation with the PR8 strain, are shown in Table II. In these experiments all embryos employed were 5 days of age. In the large majority of instances embryos which had been inoculated after death were capable of supporting the multiplication of influenza virus if they were incubated at 35°C. Moreover, dead embryos held at room temperature for 3 days before inoculation or 4 days there-hours, then at room temperature for 7 days, and finally at 37°C for 16 days, they were able to culture living cells from the embryo or its membrane in plasma clots.

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[5] Pearson, H. E., *J. Bact.*, 1944, **48**, 369.

It seemed of both practical and theoretical interest to determine whether influenza virus would multiply in dead chick embryos. A number of experiments were carried out in which the time and temperature, at which embryos were held, were varied over wide ranges. The results obtained indicate that the PR8 strain of influenza A virus is capable of multiplication in the tissues of dead chick embryos if incubation at 35°C is sufficiently prolonged. They show also that the virus multiplies in dead embryos to an extent comparable to its multiplication in the living embryo.

Materials and Methods. The PR8 strain of influenza A virus was employed. It was cultivated in the allantoic sac of 9-day embryos which were incubated at 39°C before inoculation and at 35°C thereafter. Infected allantoic fluid gave hemagglutination and embryo infectivity titers of the order of 1:2000 and $10^{-8.5}$, respectively. Infected fluid was diluted in sterile nutrient broth; 0.2 cc of either a 10^{-3} or 10^{-6} dilution was employed as the inoculum.

The embryos of White Leghorn eggs were used. They were incubated at 39°C for periods ranging from 5-10 days. In certain experiments, after inoculation of the virus into the allantoic sac, embryos were held at room temperature for 3-10 days, and thereafter incubated at 35°C for 2-10 days. In other experiments, normal embryos were chilled at 4°C for either 20 or 96 hours, some of the former then held at room temperature for 3 days, after which they were inoculated into the allantoic sac with virus. Following this, the embryos were held at room temperature for an additional period of 2-10 days, and finally incubated at 35°C for 2-10 days. Groups of 2-3 embryos were employed and a variety of different experimental conditions were used. Specimens of allantoic fluid were removed repeatedly from individual embryos, usually at intervals of 2 days. In this way it was possible to follow the multiplication of the virus in individual embryos.

Measurement of the concentration of virus in the extra-embryonic fluid was carried out by the hemagglutination technique as described previously.[3] Washed chicken RBC in a final concentration of 0.25% were used. The allantoic fluid dilution-RBC mixtures were held at room temperature for one hour. The titration end point was taken as that dilution which gave a 2+ reaction. Hemagglutination-inhibition tests[3] with specific immune rabbit sera were carried out with a constant dilution of serum capable of inhibiting hemagglutination by 128 units of the homologous virus.

Virus infectivity titrations were carried out as described previously.[4] Serial tenfold dilutions of fluid were inoculated into the allantoic sac of 9-day chick embryos and, after incubation at 35°C for 48 hours, the allantoic fluids were removed and tested by the hemagglutination procedure. The infectivity end point was calculated in the usual way.

Results. It was found that the time required to kill embryos at room temperature varied somewhat with their age. Thus, 5-day embryos held at room temperature for one day were almost always dead; 8-day embryos held for 2 days were usually dead, and invariably when held for 4 days; 10-day embryos held for 3 days were usually dead, and invariably when held for 4 days. Only 5-day embryos were held at 4°C and in every instance it was found that 20 hours at this temperature resulted in death. Embryos were considered to be dead when spontaneous movements had ceased, blood vessels showed no pulsation and no further development occurred.

The results obtained with embryos held for long periods at room temperature after inoculation with the PR8 strain are shown in Table I. It was found that multiplication of the virus did not occur when embryos were kept at room temperature for periods up to 8 days and not incubated at 35°C thereafter. However, with inoculated embryos which had been held at room temperature for 7-10 days, and then incubated at 35°C for periods ranging from 2-10 days, definite evidence of mulchick embryo tissue culture medium. It was surprising, however, to find that when dead embryos were incubated at 35°C for a sufficient period the virus titer became much higher than is found in liquid tissue culture.[6] The evidence obtained does not suggest that the virus is capable of multiplying in the absence of living cells. It has been demonstrated clearly[1,2] that in embryos killed by chilling at 4°C or by prolonged storage at room temperature, living cells are present. Presumably these cells carry on their usual metabolic functions when held at 35°C. It seems probable that the long period of incubation necessary for the development of maximal virus titers may be related to and dependent upon the multiplication of such living cells in both the dead embryo and its membranes. In this connection it will be recalled. that when embryos were frozen at −30°C they were thereafter incapable of supporting viral multiplication.

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[3] Hirst, G. K., *J. Exp. Med.*, 1942, 75, 49.

[4] Hirst, G. K., *J. Immunol.*, 1942, 45, 285.

It is apparent that these results may be of practical usefulness, especially in field work where attempts are made to recover viruses from infected persons or animals. In the case of influenza virus, at least, it seems evident that it is not necessary to observe special precautions with chick embryos. When adequate laboratory facilities are lacking, dead embryos which have been stored for considerable periods may prove satisfactory for the recovery of the infectious agent.

Summary. Chick embryos killed by prolonged storage at room temperature or 4°C are capable of supporting the multiplication of influenza virus upon prolonged incubation at 35°C.

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[6] Weller, T. H., and Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 124.

16989 P

Relation of Vitamin B_{12} to Liver Basophilia.*

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Vitamin B_{12} has been reported to stimulate the growth of chicks on a vegetable protein diet and of certain lactobacilli.[1,2] When it was later found that the nucleoside thymidine can replace vitamin B_{12} in the medium of these microorganisms, it was postulated that the vitamin acts as an enzyme in the synthesis of nucleosides and thus of nucleotides and nucleic acids.[3,4] It was decided to test this theory by studying the formation of nucleic acids in the rat as influenced by vitamin B_{12}. Since it is generally accepted by histochemists that the basophilia reaction determines ribonucleoprotein,[5] the effect of vitamin B_{12} on the basophilia of rat livers was studied in this experiment.

Experimental. Weanling white rats whose mothers had been fed a diet in which the sole source of protein was soybean oil meal were used in this investigation. When the young were 21 days old they were removed from the mothers and divided into 3 groups. Groups 1 and 2 received a diet of the purified type in which soybean oil meal was the sole source

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* Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Agricultural Biochemistry.

[1] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. B. C.*, 1948, **174**, 1047.

[2] Shorb, Mary L., *Science*, 1948, **107**, 397.

[3] Wright, L. D., Skeggs, Helen R., and Huff, J. W., *J. B. C.*, 1948, **175**, 475.

[4] Shive, W., Ravel, Joanne M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

[5] Demsey, E. W., and Wislocki, G. B., *Phys. Rev.*, 1948, **26**, 1.

TABLE II.

Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at 4°C or at Room Temperature for Long Periods Before Inoculation.

Before inoculation			After inoculation			
4°C (hr)	Room temp. 22-25°C (days)	Inoculum 0.2 cc intra-allantoic (dilution)	Room temp. 22-25°C (days)	35°C (days)	No. embryos*	Allantoic fluid hemagglut. titer†
20	—	10^{-3}	4	2	3	64,64,64
”	—	”	”	6	”	512,256,256
”	—	”	”	10	”	128,128,64
”	—	”	9	2-6	”	0,0,0
”	—	10^{-6}	4	2	”	0,0,0
”	—	”	”	4	”	C,2048,64
”	—	”	”	10	”	C,2048,2048
”	—	”	7	3-9	”	C,0,C
”	—	”	10	2-5	”	C,0,C
—	4	10^{-3}	4	3	”	256,0,64
—	”	”	”	6	”	32,1024,128
—	”	”	8	3	2	64,0
—	”	”	”	7	”	16,C
20	3	”	4	3	3	64 0,0
”	”	”	”	6	”	512,C,0
”	”	”	”	10	”	512,C,C
”	”	”	8	3-7	”	0,0,0
96	—	”	—	2	”	0,0,8
”	—	”	—	5	”	512,512,1024
”	—	”	—	11	”	2048,2048,2048
”	—	”	2	3-9	”	0,0,0
”	—	”	4	3-10	”	0,C,C
”	—	”	8	3-7	”	0,0,0

* All embryos were 5 days of age when inoculated.

† Hemagglutination titer of allantoic fluid from individual embryos.

C = Bacterial contamination.

after supported viral multiplication. Maximum hemagglutination titers comparable to those obtained with live embryos were demonstrated with dead embryos when incubation at 35°C was carried out for 4-6 days. Quantities as small as 10^2 E.I.D. of virus were capable of initiating multiplication in such embryos. It is of interest that even when embryos were held at 4°C for as long as 96 hours, they were still capable of supporting multiplication of the virus. It should be emphasized that such embryos had been dead for at least 3 days before inoculation.

In other experiments embryos were frozen at –30°C and held for 20 hours. They were then thawed, inoculated with a 10^{-3} dilution of virus and incubated at 35°C for prolonged periods. Under these conditions no evidence of multiplication was obtained in any instance.

The extra-embryonic fluid obtained from dead embryos had a gross appearance very different from that which is withdrawn from live embryos. In most instances the fluid was cloudy or turbid and frequently it contained some yolk, albumin, or both. As might be expected, hemagglutination reactions obtained with such fluids were somewhat abnormal, especially in the lower dilutions. However, in the higher dilutions hemagglutination appeared to be nearly normal and the end point was usually determined easily.

Discussion. Multiplication of influenza virus in the tissues of dead chick embryos is probably analogous to its multiplication in

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Hypothromboplastinemia Following Total Body Irradiation.

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(Introduced by R. W. Heinle.)
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Allen and Jacobson[1] published a report concerning the hemorrhagic syndrome associated with total body exposure to ionizing irradiation. They were able to demonstrate a circulating anticoagulant which was indistinguishable from heparin. A more detailed study of the same and similar experiments was published by Allen, Sanderson, Milham, Kirschon, and Jacobson.[2] These studies are of considerable importance with respect to the effect of irradiation upon the human being and also because of their relation to idiopathic thrombocytopenic purpura and thrombocytopenia secondary to aplasia or destruction of the bone marrow. Interest has been stimulated among radiologists, investigators working in the field of atomic irradiation, hematologists, and various others concerned with the coagulation of blood.

The syndrome that Allen and his associates produced in the dog with a total body exposure of 450 r (LD-100%) consisted principally of extensive hemorrhage throughout all organs of the body, neutropenia, thrombocytopenia, anemia, prolongation of the clotting time of whole blood, and prolongation of the bleeding and clot retraction times. Prothrombin times were within normal limits up to 24 hours prior to death. Blood fibrinogen, calcium, phosphorus, and magnesium levels were normal. The animals succumbed on an average of 11 days following irradiation. This syndrome resembles that of idiopathic thrombocytopenia with the exception of the neutropenia and prolonged clotting time. The decreased coagulability of the blood was thought to be due to a circulating heparin-like anticoagulant. This was demonstrated by prolonging the clotting time of normal plasma by the addition of plasma from an irradiated animal and by *in vivo* and *in vitro* titrations of the irradiated blood with toluidine blue and protamine sulphate.

Kohn and Robinett[3] reported no change in the clotting time of rats subjected to 1,000-1,250 r, which was 125-150% of the LD-50%. By administering heparin to the rats, the LD-50% could be reduced about one-third. Allen and his associates routinely used the LD-100%.

Our interest in this problem arose in an attempt to determine whether the reputed decreased coagulability of the blood following irradiation of the dog was due to circulating heparin or an increase of the heparin cofactor. Our experiments have demonstrated no evidence of heparinemia or increase in the heparin cofactor. A definite change in the amount of thromboplastic protein that could be extracted from plasma subjected to high-speed centrifugation was found.

Method. Studies were carried out prior to irradiation and for the most part, every other day after irradiation. Determinations consisted of the hemoglobin, erythrocyte count, leucocyte count, platelet count (blood smears, 2.5% sodium citrate), clotting time,[4] clot retraction, heparin cofactor,[5] protamine titration where indicated, and thromboplastin assay.

Healthy mongrel dogs were subjected to total body irradiation with 450 r (LD-100%) from a 220 K.V.P. machine at 15 milliamperes through a 0.5 mm copper filter and a 1.0 mm aluminum filter. H.V.L. was 1.2 mm

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[1] Allen, J. G., and Jacobson, L. O., *Science*, 1947, **105**, 388.

[2] Allen, J. G., Sanderson, M., Milham, M., Kirschon, A., and Jacobson, L. O., *J. Exp. Med.*, 1948, **87**, 71.

[3] Kohn, H. I., and Robinett, P., *Oak Ridge National Laboratory*, 1948, unclassified.

[4] Lee, R. I., and White, P. D., *Am. J. Med. Sci.*, 1913, **145**, 495.

[5] Holden, W. D., Cole, J. W., and Davis, J. H., Jr., *Surg., Gyn., and Obs.*, 1949, in press.

TABLE I.
Relation Between Diet and Basophilia.

Group	Rat No.	Dietary supplement	Gain, g per day	Basophilia, grade
1	1	None, negative control	0.8	0
	2	” ” ”	1.2	0
	4	” ” ”	1.0	+
	5	” ” ”	2.5	0
	22	” ” ”	0.9	+
	28	” ” ”	1.3	0
	29	” ” ”	0.6	0
	36	” ” ”	1.0	0
2	3	B_{12}	4.2	+++
	6	”	2.4	+++
	24	”	4.3	+++
	39	”	4.4	++
3	27	Liver	5.1	++++
	34	”	4.0	++++

of protein. Vitamin B_{12}[†] was included in the diet of Group 2 at a level of 440 μg per kilo. Group 1, the diet of which was not supplemented, served as the negative control. For Group 3, the diet was modified by the introduction of 4.2 g of dried liver containing 2.5% protein per 100 g of diet at the expense of the soybean oil meal. All diets contained 22% protein and included vitamins and salt mixture in optimal amounts. The pups were allowed to feed and drink *ad libitum* and were sacrificed at the end of the 21-day experimental period.

Segments of liver were removed immediately after the animals were sacrificed, fixed in Carnoy's fluid, dehydrated through alcohol, and sectioned at 8 μ. The sections were stained with eosin and methylene blue.

Results. An arbitrary scale was decided upon to evaluate the extent of the basophilic staining. Livers which showed no cytoplasmic basophilia were graded 0, and those which exhibited minimal basophilia were graded +. Moderate concentrations were graded ++; sections with more intensive staining were graded +++; and those sections whose cytoplasm was replete with basophilic staining were graded ++++.

Table I shows that rats which received vitamin B_{12} or dried liver grew at a markedly greater rate than those which received no supplement. Furthermore, the animals which gained slowly were characterized by little or no hepatic basophilia, whereas those which gained rapidly showed extensive cytoplasmic basophilia in the liver cells.

The review by Demsey and Wislocki[5] points out that basophilia, shown to be ribonucleoprotein, is intimately related to protein synthesis. It would thus seem that when animals are deprived of vitamin B_{12} the formation of nucleoprotein (through the nucleoside) is limited, and consequently protein synthesis in the body would be retarded.

Summary. The growth of weanling white rats was stimulated by inclusion of vitamin B_{12} or of whole dried liver in the diet. Rats which received no B_{12} or liver grew poorly and showed little or no liver basophilia, whereas those which received B_{12} or liver grew well and showed considerable cytoplasmic basophilia in their liver cells.

[†] Vitamin B_{12} concentrate was kindly supplied by Merck & Company, Rahway, N. J.

TABLE II.
Average of Blood Determinations on the Day Before Irradiation and the Day of Death.

	Before irradiation	Day of death
Hb. g/100 ml	13.4	10.1
RBC/c.mm. × 10^6	6.5	3.8
WBC/c.mm.	12,300	175
Platelets/c.mm.	236,000	6,630
Clotting time (min.)	8	8
Clot retraction	Normal	0
Heparin cofactor. % thrombin destroyed	89	83
Clotting time (sec.) supernatant plasma	147	440
Prothrombin time (sec.)	14.8	17.4
Clotting time. (sec.) supernatant plasma + sediment	70	107

anism or hematologic picture that could be subjected to a critical analysis. The other 3 dogs survived 9, 10, and 11 days respectively and displayed the same clinical changes and similar alterations in the studies of their blood. At autopsy the following findings were observed. In general, multiple focal pulmonary and epicardial hemorrhages were present. Submucosal hemorrhages were scattered throughout the gastrointestinal tract, and were routinely present in the urinary bladder. Small areas of bronchopneumonia were present in one dog, while in another, pneumonic consolidation was widespread. A hemothorax (50 ml) was present in one dog and a bilateral pleural effusion in another.

Table I shows the studies performed during the course of one experiment, while Table II shows the average determinations of 3 experiments before irradiation and on the day of death. The significant findings so far as the hemorrhagic tendency is concerned are the reduction in thrombocytes, the increase in the clotting time of the supernatant plasma, and the increase in the clotting time of the supernatant plasma after the addition of the suspended sediment (Fig. 1). These latter 2 determinations represent thromboplastic protein in the blood stream and indicate a definite diminution following irradiation. The gradual reduction of thromboplastic activity in the plasma parallels the reduction in thrombocytes, but it cannot be stated from these observations that the thromboplastic protein was derived from the thrombocytes. The following determinations demonstrate that the prolongation of the clotting time was not due to the presence of an anticoagulant or the depletion of either prothrombin or fibrinogen.

Protamine sulphate[†] in amounts from 0.02 mg to 0.06 mg in 0.1 ml of physiological saline was added to 0.1 ml of the supernatant plasmas after their clotting times had become prolonged. After adding 0.1 ml of 0.022 M calcium chloride solution, there was in no instance a shortening of the control clotting time. In other experiments[7] plasma obtained from heparinized blood showed a return of the prolonged clotting time to normal or near normal values after the addition of protamine sulphate. The above titrations suggest that no anticoagulant subject to neutralization by protamine was effecting the deceleration of clot formation in the supernatant plasma.

When 0.1 ml of commercial thromboplastin and 0.1 ml of 0.022 M calcium chloride solution were added to the supernatant plasma, a satisfactory estimate of the available prothrombin was determined. Clotting times varied from an average of 14.7 seconds before irradiation to 17.4 seconds on the day of death. Although this represents a slight reduction in the amount of prothrombin in the supernatant plasma, it hardly accounts for either the marked prolongation of the clotting time of the recalcified supernatant plasma or the hemorrhagic manifestations exhibited by the animals.

The fact that a tenacious solid clot could always be obtained from the recalcification of the supernatant plasma indicated that an adequate quantity of fibrinogen was present. This was further demonstrated by adding 10

[†] The Upjohn Company.

[7] Portmann, A. F., and Holden, W. D., to be published.

TABLE I.
Hematologic and Coagulation Changes in Exp. No. 5.

Date	11/22	11/24	11/26	11/29	12/1	12/3	12/6	12/8
Hb. g/100 ml	16.0		15.5	15.0	13.5	13.0	11.0	9.0
RBC/c.mm. $\times 10^6$	6.56		6.23	6.03	6.12	5.59	4.02	3.62
WBC/c.mm.	15,275		10,700	2,000	600	760	175	50
Platelets/c.mm. $\times 10^3$	308		236	223	110	16	4	
Clotting time, min.	7	Irradiated 450 r.	5	5	9	9	9	9
Heparin cofactor. % thrombin destroyed	92.2		95	93.5	92	92.3	94.9	85
Clotting time supernatant plasma (sec.)	138		190	283	403	348	510	600
Clotting time (sec.) thromboplastin + supernatant plasma	24		21	22	29	22		
Clotting time (sec.) suspended sediment and supernatant plasma	84		89	87	120	120	132	
Clot retraction	Normal							None

of copper. Irradiation was delivered at a rate of 6-7 r per minute.

Chargaff and West[6] demonstrated that a sediment exhibiting thromboplastic activity could be removed from oxalated plasma subjected to high-speed centrifugation. The clotting time of the supernatant plasma was an index of the amount of thromboplastic protein remaining after centrifugation. The clotting time of the supernatant plasma after portions of the sediment were added to it also indicated the amount of thromboplastic activity in the original plasma. In this way, minute changes in the thromboplastic protein, measured in gammas, could be demonstrated.

The following procedure was used to assay the thromboplastic protein of the plasma. Twenty ml of blood were removed from the jugular vein with as little tissue trauma as possible and quickly mixed with 2.0 ml of a 0.1 M sodium oxalate solution. The blood was centrifuged at 4,000 r.p.m. (2,400 g) for 20 minutes at a temperature of 4°C in a refrigerated International centrifuge. The supernatant plasma was carefully pipetted off, and this was centrifuged for 150 minutes at 20,000 r.p.m. (33,000 g) at the same temperature. The plasma was decanted. The sediment remaining in the base of the centrifuge tube was then suspended in normal saline. The amount of saline used was one-tenth the volume of supernatant plasma. To 0.1 ml of the supernatant plasma was added 0.1 ml of normal saline, and the mixture was placed in a water bath at 37°C for one minute. One-tenth milliliter of 0.022 M calcium chloride solution was added and a stopwatch started. The clotting time was determined in seconds. When short clotting times were obtained, solid clot formation occurred and this was used as the end point. When the clotting time was prolonged, the formation of a solid clot was preceded by fibrin strands. End points were indicated by the first appearance of the latter. One-tenth milliliter samples of the supernatant plasma were then added to saline and various concentrations of a suspension of thromboplastin* and the clotting times determined after incubation for one minute at 37°C and recalcification. The suspension of thromboplastin was prepared by mixing 50 mg of the thromboplastin with 12 ml of normal saline and heating it for 10 minutes at 50°C. Following the above procedure, 0.1 ml of the suspended sediment was added to 0.1 ml of the supernatant plasma. The mixture was incubated for one minute at 37°C and then quickly recalcified by adding 0.1 ml of the 0.022 M calcium chloride solution.

Results. Five dogs were irradiated. One lived 5 and another 7 days, and neither showed any changes in the clotting mechactivity of hepatic polygonal cells, or of Kupffer cells.

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[6] Chargaff, E., and West, R., *J. Biol. Chem.*, 1946, **166**, 189.

* The Maltine Company.

In order to study this problem, advantage has been taken of the fact that tetraiodotetrachlorfluorescein (Rose Bengal), a dye introduced for the study of liver function by Kerr *et al.*[3] exhibits a soft reddish-orange fluorescence in the long ultraviolet spectrum (3150-3650 Å). Tissues stained with 1-2% aqueous solutions of this dye emitted the same characteristic fluorescence, which was readily observed through a yellow eyepiece filter on an ordinary microscope by ultraviolet light (Source—General Electric H-4 medium pressure mercury vapor lamp) passed through glass condensers, Woods filter, 5% copper sulfate solution, glass substage condenser, and glass slides. The fluorescence was not intensified when observed in an all-quartz optical system, and remained unaffected by several hours' exposure to the illumination described above.

Aqueous solutions (1-2%) were prepared from standard bacteriological Rose Bengal powder;[†] these were autoclaved, and doses varying from 20 to 100 mg were injected into the ear veins of adult rabbits lightly anesthetized with ether. Liver and splenic biopsies in 20 such rabbits were obtained from 1 to 15 minutes after injection. The tissues were immediately placed in saline, then cut on the freezing microtome into sections 15 μ thick. These were floated on water, mounted on ordinary glass slides, and examined within 10 minutes after mounting. The characteristic sustained fluorescence due to Rose Bengal was noted constantly in the hepatic polygonal cells. In biopsies made within the first 3-4 minutes following injection of the dye, the polygonal cell fluorescence was somewhat less intense than in those biopsies made at 10 minutes after injection. Along the endothelial lining of the sinusoids, where Kupffer cells are normally found, no such fluorescence was observed. One constantly saw, however, in these locations as well as in the periphery of some polygonal cells, the greenish-white, rapidly fading fluorescence ascribed by Popper and Greenberg[4] to Vitamin A. Grossly, in ordinary white light, the livers were definitely reddish. In 16 rabbits, the color of the spleen differed in no way from that of uninjected controls, but in 4 rabbits it was slightly pink. Such faint orange fluorescence as was seen in splenic sections did not appear to be located in the parenchyma.

Two anesthetized human subjects undergoing laparotomy were subjected to liver biopsy just prior to and 10 minutes following intravenous injection of 150 mg of Rose Bengal (2% solution). These biopsy tissues were prepared exactly as described above. They showed the same picture as did the rabbit livers—the orange fluorescence due to Rose Bengal in the hepatic polygonal cells, but nowhere else.

These observations provide evidence that the dye Rose Bengal is taken up by, and stored in the hepatic polygonal cells rather than in the Kupffer cells of the liver. Whether the Kupffer cells participate in the removal of the dye from the blood, although unlikely, cannot with certainty be excluded by the technique of fluorescence microscopy. All one can say is that, during a period of 1 to 15 minutes after injection of the dye, the fluorescence due to Rose Bengal is not observed in those areas where Kupffer cells are commonly found. In this respect these findings are similar to those of Grafflin[5] on the handling of fluorescein by the frog liver. Other evidence obtained in this laboratory by hepatic venous catheterization of normal man[6] shows clearly that the hepatic uptake of Rose Bengal is slowed or blocked by simultaneously-administered Bromsulfalein. It is suggested, therefore, that the removal of both these dyes is normally a function of the hepatic parenchymal cells rather than of the Kupffer cells.

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[2] Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., *J. Clin. Invest.*, 1945, **24**, 890.

[3] Kerr, W. J., Delprat, G. D., Epstein, N. N., and Dunievitz, M., *J. A. M. A.*, 1925, **85**, 942.

[†] National Aniline Company.

[4] Popper, H., and Greenberg, R., *Arch. Path.*, 1941, **32**, 11.

[5] Grafflin, A. T., *Seventh Conference on Liver Injury*, p. 75, Josiah Macy, Jr., Foundation, N.Y., 1948.

[6] Mendeloff, A. I., and Ingelfinger, F. J., unpublished data.

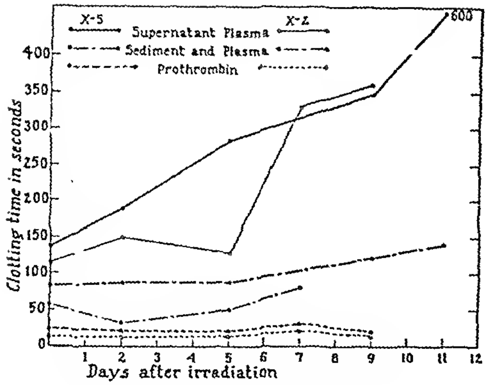

FIG. 1.

Graphic representation of the changes in supernatant plasma, sediment and plasma, and prothrombin in two experiments.

units of thrombin‡ to 0.1 ml of the supernatant plasma and 0.1 ml of 0.9% sodium chloride solution and obtaining satisfactory clots in less than 5 seconds.

Clotting times performed on whole blood throughout all experiments showed no significant changes. Determinations of the heparin cofactor likewise showed no change of importance. The failure to demonstrate any prolongation of the clotting time of the blood is at variance with the observations of Allen and his associates.[1,2] We have no satisfactory explanation of this. The determination of the clotting time of whole blood by any of the various test tube methods is notoriously difficult to perform and interpret. For this reason special precautions were taken. The same physician drew all the blood and performed all the clotting times under the same circumstances. We do not wish to deny that heparinemia may exist after irradiation of this character, but up to the present we have been unable to satisfy ourselves that it exists, whereas we have been able to demonstrate a hypothromboplastinemia and thrombocytopenia which explain the clinical manifestations exhibited by these animals.

Conclusions. 1. A hemorrhagic syndrome in dogs was produced by total body irradiation (450 r). Manifestations consisted of multiple areas of hemorrhage in the pulmonary, gastrointestinal, and urinary systems, anemia, leucopenia, thrombocytopenia, prolongation of clot retraction, and hypothromboplastinemia.

We wish to thank Mrs. Ruth Stavitsky for her technical assistance.

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‡ Parke, Davis and Company.

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Fluorescence of Intravenously Administered Rose Bengal Appears Only in Hepatic Polygonal Cells.

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Although certain intravenously administered dyes have been employed in the study of hepatic function over the past 3 decades, the exact mechanisms by which they are removed from the blood are poorly understood. Earlier work indicated that the entire reticulo-endothelial system participated in the removal of these substances. This concept is still widely prevalent, although more recently indirect evidence suggests that the normal liver alone is responsible for the uptake of such dyes as the disulfonate of phenoltetrabromphthalein (Bromsulfalein).[1] Now that hepatic blood flow in man is being estimated by a technique based on the extraction of Bromsulfalein by the liver,[2] it is essential to determine whether the removal of this dye, or of others similarly handled, measures the

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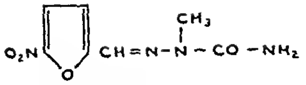
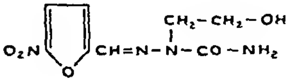
* Postdoctorate Research Fellow of the National Institutes of Health.

[1] Ingelfinger, F. J., *Bull. New Eng. Med. Cen.*, 1947, 9, 25.



5-nitro-2-furaldehyde-2-β-methyl semicarbazone



5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone

FIG. 1.

Structural formulae of 5-nitro-2-furaldehyde-2-β-hydroxethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone.

2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methylsemicarbazone (Fig. 1), in 500 μg quantities, completely inhibited acid production in 10-cc aliquots of paraffin stimulated saliva in 90.5 and 85% of the samples respectively. A significant but incomplete inhibitory effect was observed in all other instances. Both compounds proved to be bactericidal against an oral strain of the Lactobacillus acidophilus, when added in amounts of 3.5 mg and above to yeast extract dextrose broth inoculated with the test organism.

To investigate the possibility that conditions in the mouth not reproducible *in vitro*, such as the absorption potential of the oral mucous membranes, tissue enzymes and the migration of phagocytes to the oral cavity, may interfere with the antibacterial action of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone on the organisms associated with dental caries, it was decided to determine whether these compounds retained their effectiveness as inhibitors of acid production when added *in vivo* to the saliva of persons highly susceptible to dental decay.

Materials and Methods. Sixty-nine patients, each of whom had clinical and laboratory evidence of active dental caries, were selected for this study. The clinical examination was made with a mouth mirror and exploring tine and was supplemented in most instances by bite wing or full mouth roentgenograms. Laboratory evidence of prevailing dental caries activity was established by analyzing samples of saliva by the methods of Hadley,[5] Fosdick, Hansen, and Epple,[6] and Dreizen, Mann, Cline and Spies.[7]

Method of Study. Two samples of stimulated saliva comprising 20 cc and 15 to 20 cc respectively were collected in sterile flasks from each patient. An untreated paraffin block served as the salivary stimulant for the first sample and a paraffin block containing 750 μg or 1,000 μg of the test compounds served as the secretory stimulant for the second. The collection time for each sample ranged from 10 to 20 minutes with a 15 minute interval between samples. Paraffin blocks containing 750 μg of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone, 1,000 μg of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone, 750 μg of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone, and 1,000 μg of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone were used for 20, 23, 7 and 19 patients respectively.

A 10-cc aliquot of the saliva sample obtained with the untreated paraffin block was tested immediately for soluble calcium by the method of Fosdick, Hansen, and Epple.[6] Ten-cc aliquots of the samples obtained with the nitrofuran-treated and untreated paraffin blocks were added to sterile brown vials containing dextrose and tricalcium phosphate. The vials were sealed, shaken, incubated and agitated in a water bath maintained at 37°C. Following a 4-hour period of incubation and agitation, the amount of soluble calcium contained in each of these aliquots was determined. The difference in calcium values between the incubated and unincubated aliquots of saliva for each patient was regarded as a measure of the degree of acid production.

Observations. The effect of the *in vivo* addition of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone on acid formation in the

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[4] Main, R. J., *J. Am. Pharm. Assn.*, 1947, **36**, 317.

[5] Hadley, F. P., *J. D. Res.*, 1933, **13**, 415.

[6] Fosdick, L. S., Hansen, H. S., and Epple, C., *J. A. D. A.*, 1937, **24**, 1275.

[7] Dreizen, S., Mann, A. W., Cline, J. K., and Spies, T. D., *J. D. Res.*, 1946, **25**, 213.

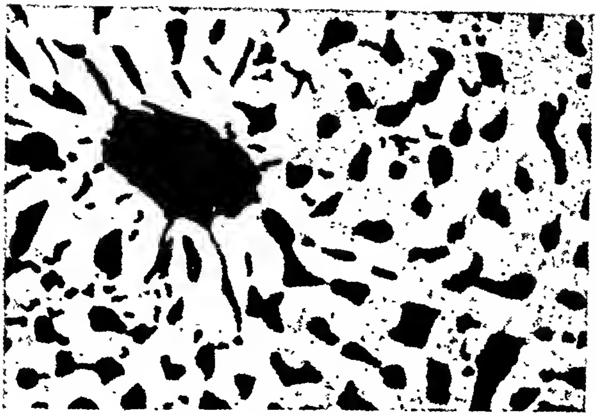

FIG. 1.
Low-power photomicrograph of rabbit liver (300 ×, enlarged 2 ×), fixed in formalin and examined unstained by ultraviolet light. The print has been retouched to emphasize the contrast between non-fluorescent sinusoids and fluorescent hepatic trabeculae, and to remove artifacts which displayed light yellow or green fluorescence.

Summary. Intravenously-administered Rose Bengal can be visualized by fluorescence microscopy in the livers of rabbits and humans. The fluorescence is seen in hepatic polygonal cells, but not in Kupffer cells. It is suggested that the polygonal cells of the normal liver are responsible for the storage of Rose Bengal, and, by analogy, Bromsulfalein.

The author acknowledges with thanks the advice and criticism of Doctors F. J. Ingelfinger, F. Sjostrand, and J. Scott.

16992

Effect of *in vivo* Addition of Selected Nitrofurans on Acid Production in Human Saliva.*

SAMUEL DREIZEN, HENRY I. GREENE, AND TOM D. SPIES.
From the Department of Nutrition and Metabolism, Northwestern University.

Investigation of the relation between nutritional status and dental caries has been a major study at the Nutrition Clinic of the Hillman Hospital, Birmingham, Alabama, since 1941. During the past year an extensive study was made of the effect of the *in vitro* addition of selected nitrofuran compounds on acid production in human saliva, and on the growth and acid production of a pure culture of a strain of the Lactobacillus acidophilus recovered from a carious lesion.[1] The antibacterial action of the nitrofurans has been reported by a number of investigators.[2-4] Our findings showed that 5-nitro-

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* Northwestern University Studies in Nutrition at the Hillman Hospital, Birmingham, Ala.

These studies were supported by grants from Eaton Laboratories, Inc.

We wish to thank Mrs. Thelma S. Brummal for her aid in making the caries activity determinations.

[1] Dreizen, S., Greene, H. I., and Spies, T. D., in press.

[2] Dodd, M. C., Hartmann, F. W., and Ward, W. C., *Surg., Gynecol., and Obstet.*, 1946, **83**, 73.

[3] Dodd, M. C., and Stillman, W. B., *J. Pharm. and Exp. Therap.*, 1944, **82**, 11.

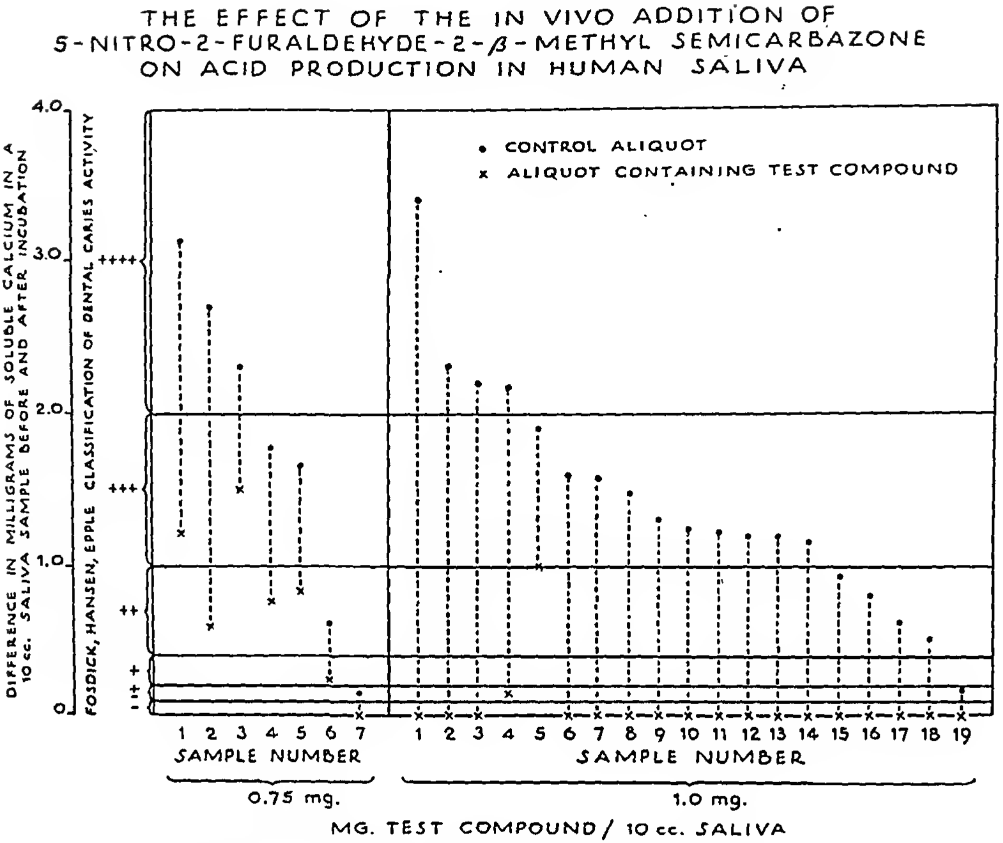

FIG. 3.

The sample numbers are arranged in the order of decreasing dental caries activity in the control aliquot and not in the order in which the samples were collected.

duction in the saliva of caries active patients. The difference may be attributed in part to the unavoidable loss of some of the test material by admixture with the paraffin used as the secretory stimulant, by swallowing, and by retention in the sheltered areas and crevices of the oral cavity.

Each of the test compounds is but slightly soluble in water, the solubility being 1:900 for 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone, and 1:3000 for 5-nitro-2-furaldehyde-2-β-methyl semicarbazone. It appears, therefore, that the actual inhibitory activity of these compounds may be attributed to a fraction of the concentrations employed in this and in the previous study. The difference in the extent of the solubility of the test compounds may also account for the greater effectiveness of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone as an inhibitor of acid production in the saliva when added in 750 μg quantities *in vivo*, as compared with a similar amount of the less soluble 5-nitro-2-furaldehyde-2-β-methyl semicarbazone.

Summary and Conclusions. The incorporation of 1,000 μg quantities of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone in paraffin blocks used as salivary stimulants completely prevented acid production in 38 of the 42 saliva samples obtained from caries active individuals. A significant partial reduction in acid formation occurred in the remaining 4 cases. The findings indicate that 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone are not rapidly destroyed or absorbed by the tissues and fluids of the oral cavity.

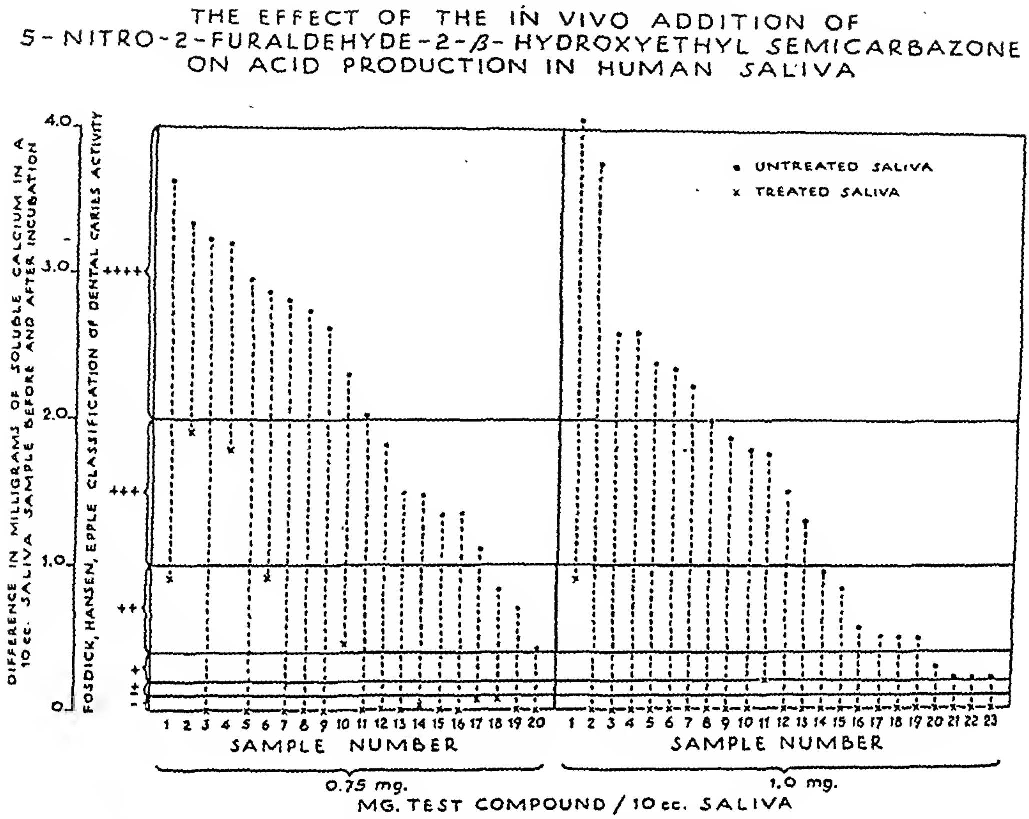

Fig. 2.

The sample numbers are arranged in the order of decreasing dental caries activity in the control aliquot and not in the order in which the samples were collected.

saliva of caries active individuals is shown in Fig. 2. When the compound was added in 750 μg amounts, acid production was completely inhibited in 15 (75%) of the 20 samples tested. A significant partial reduction in acid formation was observed in each of the remaining 5 samples. The *in vivo* addition of 1,000 μg amounts of this compound resulted in a complete inhibition of acid formation in 21 (91%) of the 23 samples tested. Acid production was reduced to a significant extent in the remaining 2 samples.

Fig. 3 demonstrates the results of the addition *in vivo* of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone on acid production in the saliva of caries susceptible individuals. In the 7 instances in which 750 μg quantities of this compound were added, acid formation was completely prevented in only 1 (14%) of the samples, the amount of acid formed in the untreated aliquot of this sample being negligible. Acid production was deterred, however, to a significant degree in each of the remaining samples. When the amount of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone incorporated in the paraffin blocks was increased to 1,000 μg, acid production was completely prevented in 17 (89%) of the 19 samples of saliva in which this concentration was used. A significant partial reduction in the amount of acid formed was noted in the remaining 2 samples.

Discussion. The evidence presented by this study indicates that the prevention of acid formation in saliva by 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone is not adversely affected in the presence of the tissues and fluids of the oral cavity. In each instance a slightly higher concentration of these compounds was required when added *in vivo* than *in vitro* in order to inhibit acid pro-

capsulatum as well as single strains of *Phialophora verrucosa* and *Trichophyton mentagrophytes* appeared to be increased in the presence of streptomycin. The stimulatory effect was noted as early as 48 hours with *S. schenckii* while similar results were obtained with the other organisms in periods varying from 1-3 weeks. The stimulatory effect was proportional to the concentration of streptomycin up to 2.5 mg per ml, at which level maximum growth was obtained. This effect was noted only with young actively growing cultures and not with old stored suspensions. Plate 1 depicts photographically the results obtained in representative tests.

Since streptomycin is known to be more effective at an alkaline pH, similar studies were made with *S. schenckii* and *C. immitis* in media adjusted to 4.0, 6.0, and 8.0, respectively. Although the amount of growth in the control tubes was considerably less at pH 8.0 than in the acid range, the same stimulatory effect in the presence of streptomycin noted above was observed at all 3 levels.

Summary. Streptomycin incorporated into a synthetic liquid medium enhanced the growth of laboratory strains of *S. schenckii, C. immitis, P. verrucosa, T. mentagrophytes,* and *H. capsulatum* which had not been previously exposed to this antibiotic.

16994

Phosphoprotein Phosphatase in Rat Young, Embryos, and Placentas.

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From the Toxicity Laboratory and the Department of Biochemistry,‡ University of Chicago.

Phosphoproteins appear to exist uniquely in food sources for the embryo and young (*e.g.*, casein in milk, vitellin in egg yolk, ichthulin in fish eggs). A clue to the significance of this fact was seen in the observation by Harris[1] that frog eggs contained an enzyme, designated phosphoprotein phosphatase, which released inorganic phosphorus from phospho-proteins without preliminary proteolysis. This was confirmed by Feinstein and Volk,[2] who further showed such an enzyme present also in various mammalian tissues.

The present report presents data regarding the activity of this enzyme in rat placentas, embryos, and young, all of various ages.

Methods. Dated conceptions were obtained by following vaginal smears and introducing the female to the male during an overnight period during estrus. At the desired time after conception, the pregnant female was sacrificed, and the embryos and placentas were removed, separated by rough dissection, quickly frozen in an ether-dry ice mixture, and stored under deep freeze until convenient for assay. Several placentas from each female were pooled for assay, as were 14- and 16-day embryos. Young rats were sacrificed by immersion in the freezing mixture and stored under deep freeze.

For assay, placentas and embryos were homogenized in a glass homogenizer; young rats were blended in a Waring blendor. Further details of the assay and analytical methods have been described.[2]

Results. Fig. 1 presents the phosphoprotein phosphatase activity of whole placentas, embryos, and young rats, all of varying ages.

Fig. 2 presents the activity of this enzyme per gram of tissue and per mg of protein, in

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* This report is from a thesis submitted by Murray E. Volk to the Graduate School of the University of Chicago in partial fulfilment of the requirements for the degree of Master of Science (Biochemistry).

† Present address: Research Institute, Temple University, Philadelphia, Pa.

‡ The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army, and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

[1] Harris, D. L., *J. Biol. Chem.*, 1946, **165**, 541.

[2] Feinstein, R. N., and Volk, M. E., *J. Biol. Chem.*, in press.

16993 P

Enhancement of Growth of Certain Fungi by Streptomycin.

CHARLOTTE C. CAMPBELL AND SAMUEL SASLAW. (Introduced by Maurice Landy.)
From the Department of Bacteriology, Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.

Recent reports[1-7] have shown that the growth of some streptomycin-resistant bacteria may be dependent upon or enhanced by this antibiotic. A primary requisite for this phenomenon was previous exposure of these organisms to this drug. Although it is well known that streptomycin is not effective against fungi, there have been no observations, to our knowledge, concerning the enhancement of growth of fungi by streptomycin. The incorporation of streptomycin into certain selective media for fungi[8] has been instituted for the purpose of inhibition of bacteria. Employing a synthetic liquid medium containing streptomycin, we noted that the growth of certain fungi not previously exposed to streptomycin was more luxuriant than when streptomycin was absent. To further evaluate this observation, the studies described below were conducted on available laboratory strains of fungi which also had not been previously exposed to streptomycin.

Methods. The fungi were assayed in triplicate in a synthetic liquid medium consisting of inorganic salts and 0.5% glucose* to which commercial streptomycin† was added in concentrations of 0.01 to 5.0 mg/ml of medium. The final pH of the medium was 4.0. Inocula were prepared by washing the growth from 3-weeks-old Sabouraud's cultures with sterile saline and adjusting the turbidity to match a No. 3 MacFarland nephelometer standard. The suspensions were homogenized as much as possible by vigorous shaking and pipetting, and the heavier particulate matter allowed to settle. One-tenth ml amounts were then inoculated in triplicate into the test media as well as into control tubes without streptomycin. All cultures were incubated at 28°C and examined daily for comparative growth for 4 weeks.

Results. The growth of all of 6 strains of *Sporotrichum schenckii*, 4 of 4 strains of *Coccidioides immitis*, 2 of 3 of *Histoplasma*

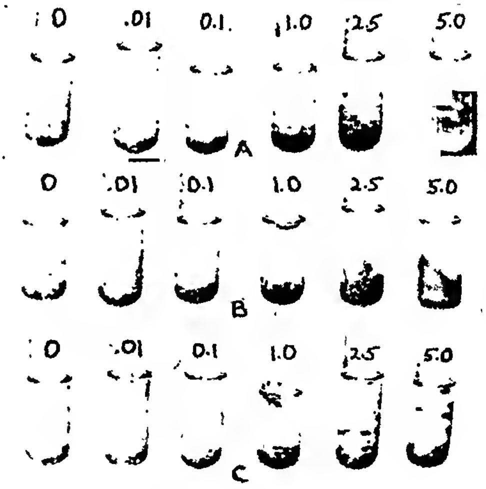

PLATE 1.
Effect of streptomycin on the growth of fungi. Each figure represents mg streptomycin/ml of medium.
A. *S. schenckii*—8th day.
B. *C. immitis*—9th day.
C. *H. capsulatum*—9th day.

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[1] Miller, P. L., and Bohnhoff, M., *Science*, 1947, **105**, 620.

[2] Kushnick, T., Randles, C. I., Gray, C. T., and Birkeland, J. M., *Science*, 1947, **106**, 587.

[3] Paine, T. F., and Finland, M., *Science*, 1947, **107**, 143.

[4] Vanderlinde, R. J., and Yegian, D., *J. Bact.*, 1948, **56**, 357.

[5] Spendlove, G. A., Cummings, M. M., Fackler, W. B., and Michael, M., *Pub. Health Rep.*, 1948, **63**, 1177.

[6] Iverson, W. P., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 586.

[7] Hobby, G. L., and Dougherty, N., *ibid.*, 1948, **69**, 544.

[8] Thompson, L., *Proc. Staff Meetings of Mayo Clinic*, 1945, **20**, 248; Littman, M. L., *Am. J. Clin. Path.*, 1948, **18**, 409.

* 4.5 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g $(NH_4)Cl$, 25 ml M/500 in N/50 HCl $FeNH_4SO_4$, 10 ml 0.4% $MgSO_4 \cdot 7H_2O$, 5.0 g glucose and distilled water to 1000 ml.

† Duplicate studies were done with Merck's streptomycin hydrochloride and Pfizer's streptomycin sulfate.

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SECTION MEETINGS

CLEVELAND	
Western Reserve University	March 11, 1949
MINNESOTA	
University of Minnesota	March 16, 1949
MISSOURI	
St. Louis University	March 14, 1949
ROCKY MOUNTAIN	
Veterans Administration Hospital, Fort Logan, Colorado	March 5, 1949
SOUTHERN CALIFORNIA	
University of Southern California	January 27, 1949
Cedars of Lebanon Hospital	February 23, 1949

16995

Antibacterial Activity of d-Usnic Acid and Related Compounds on *M. tuberculosis*.

ALFRED MARSHAK,* WERNER B. SCHAEFER, AND SRINIVASA RAJAGOPALAN.†

From New York University, College of Medicine and the Converse Memorial Laboratory, Harvard University.

Usnic acid, a lichen acid isolated over a century ago, has been the subject of active study at various schools of chemistry in respect to structure. That it is a promising antibiotic particularly antagonistic to *M. tuberculosis in vitro* and *in vivo* was discovered by Marshak and has since been confirmed *in vitro* by Stoll, Brack and Renz, Barry and more recently by Shibata and Ukita.[1-4]

The excellent antibacterial characteristics of usnic acid are somewhat offset by its poor water solubility which leads to difficulty in administration to animals and a considerable degree of toxicity. It is therefore of interest to ascertain whether or not these drawbacks could be counteracted by suitable operations on the molecular architecture of usnic acid. Accordingly, a series of derivatives and degradation products of d-usnic acid have been prepared in the hope that one or other of these compounds (No. 2-10, No. 15-18 and No. 21) might possess more advantageous pharmacological and antitubercular properties than usnic acid itself; these could conceivably arise in the animal organism as a result of the customary detoxification mechanisms. More specific attempts to prepare derivatives which

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* Division of Tuberculosis, U. S. Public Health Service.

† National Institute of Health Fellow, U. S. Public Health Service, on leave of absence from the Heffkine Institute, Bombay.

[1] Marshak, A., *U. S. Public Health Repts.*, 1947, 62, 3.

[2] Shibata, S., and Ukita, T., *Japanese Med. J.*, 1948, 1, 152.

[3] Stoll, A., Brack, A., and Renz, V., *Experientia*, 1947, 3, 115.

[4] Barry, V. C., *Nature*, 1947, 160, 800.

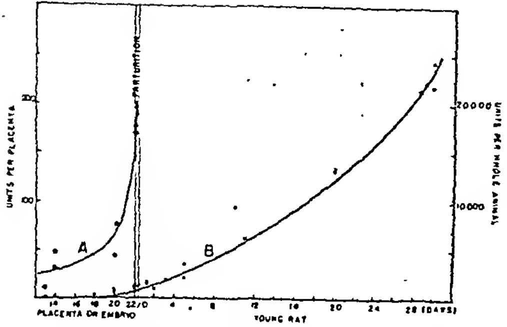

FIG. 1.

Age of (A) placenta and (B) whole animal, vs. total phosphoprotein phosphatase activity.

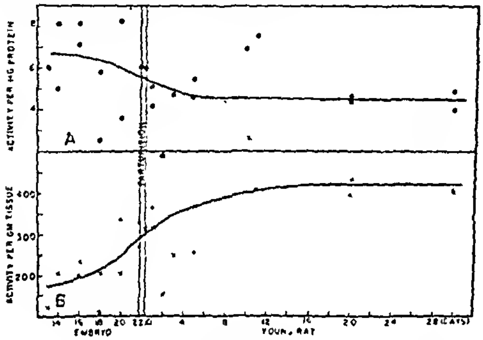

FIG. 2.

Age of animal vs. phosphoprotein phosphatase activity (A) per mg of protein and (B) per gram of body weight.

relation to the age of the embryo or young rat. Curve A, concerning activity per mg of protein, has too high a degree of scattering in the early points to warrant positive views as to the exact shape of the curve. The excessive scattering is believed due to unsatisfactory protein determinations, since the method used, that of Robinson and Hogden,[3] yielded unclear color solutions in some cases.

Fig. 3 relates placental age to enzyme activity per g tissue and per mg protein. The comment of the preceding paragraph, regarding the protein determination, is also relevant here.

Discussion. The results, particularly those of Curve B, Fig. 2, are approximately those to be expected from teleological considerations. The activity in the embryo is seen to rise, slowly at first, then more rapidly as parturition is reached. Thus the newborn animal, suddenly provided with phosphoprotein (casein) as a basic food source is simultaneously equipped to utilize it. As the animal continually increases his casein intake, the enzyme activity continues to rise. At approximately 12 days of age, the enzyme activity reaches a constant level, which is unaffected by weaning.

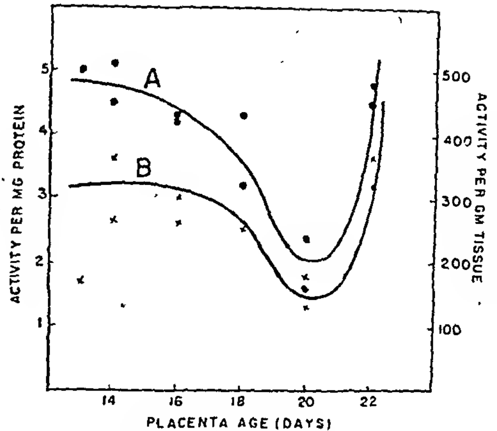

FIG. 3.

Age of placenta vs. phosphoprotin phosphatase activity (A) per gram of tissue and (B) per mg of protein.

Summary. 1. A study has been made of the level of phosphoprotein phosphatase activity in placentas, whole embryos, and whole young growing rats of various ages.

2. The total phosphoprotein phosphatase activity of the placenta and of the embryo and young rats continually rises. The activity was followed in placenta and embryo from thirteen days post-conception to parturition, and in young rats to 28 days of age.

3. The activity per gram of body weight rises slowly in early embryos, rapidly in the period immediately preceding and following parturition, and remains essentially constant during the age period of 12 to 28 days.

4. The activity per gram of placenta decreases from the 14th to the 20th day after conception, then rises again just before parturition. The preliminary decrease is due to the rate of weight gain of the placenta being greater than the rate of gain of total placenta phosphoprotein phosphatase; the reversal of direction of the curve is due to continued enzyme increase while the placenta gains essentially no further weight.

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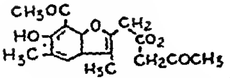
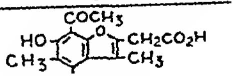
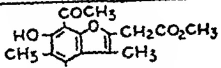
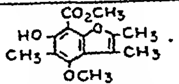
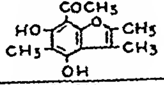
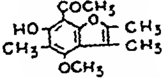
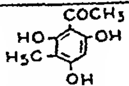
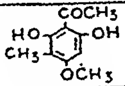
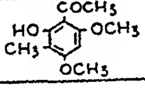
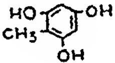
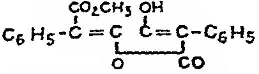
[3] Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, 1940, **135**, 707, 727.

TABLE I (Continued)

17	Decarbousnic Acid		p	10 15 20	3 3 3	3 4 4	4 4	4 4
18	Usnetic Acid		s	9 12 18	4(3) 4(3) 4(3)	4(4) 4(3) 4(3)	4(4) 4(3) 4(3)	4(4) 4(4) 4(4)
19†	Methylusnetate-4-methyl ether		p	9 12 18	3 2 2	3 4 3	4 4 4	4 4 4
20†	Methyl 2,3,5-trimethyl-4-methoxy-6-hydroxy cumarone-7-carboxylate		p	9 12 18	3 3 3	3 4 4	4 4 4	4 4 4
21	Usnetol		c	9 12 18	4(1) 4(2) 4(4)	4(1) 4(3) 4(4)	4(4) 4(4) 4(4)	4(4) 4(4) 4(4)
22†	Usnetol-4-methyl ether		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
23†	Methyl acetophloroglucinol		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
24†	Methyl acetophloroglucinol-4-methyl ether		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
25†	Methyl acetophloroglucinol-4,6-dimethyl ether		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
26†	Methyl phloroglucinol		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
27	Diacetyl usnic acid ethoxylate		s	9 12 18	3 3 3	3 3 4	4 4 4	4 4 4
28	Sodium *d*-usnate		s	10(9) 15(12) 20(18)	0(0) 0(0) 0(0)	2(0) 3(1) 3(2)	4(3) 4(4) 4(3)	4(4) 4(4) 4(4)
29	3′-Hydroxy usnanilide		p	9 12 18	0 0 0	2 3 3	4 4 4	4 4 4
30	4′-Hydroxy usnanilide		p	9 12 18	4* 3 3	2 2 3	4 4 4	4 4 4
31	4′-Dimethyl amino-usnanilide		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
32	N,β-Carboxyethyl usnamide		s	10 15 20	1 2 1	3 4 4	4 4 4	4 4 4
33	*l*-usnamido napthalene-5-sulfonic acid		s	10 15 20	4 4 4	4 4 4	4 4 4	4 4 4
34	Vulpinic acid	 $C_6H_5-\overset{CO_2CH_3}{C}=\underset{O}{C}\ \overset{OH}{C}=\underset{CO}{C}-C_6H_5$	s	10 15 20	1 2 2	3 3 3	4 4 4	4 4 4

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* Suspension slightly turbid due to low solubility of drug. This may account for higher density readings.
† Samples of these were furnished by Prof. Alexander Robertson, F.R.S., to whom our grateful thanks are due.
‡ These differed in melting points from those previously recorded in literature, but gave correct analytical figures.
Solubility: s—solution, c—colloid, p—suspension.

TABLE I.

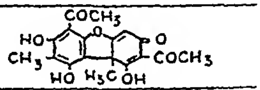
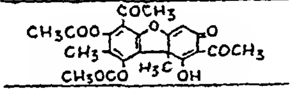
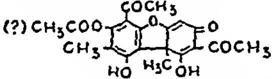
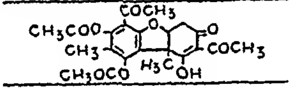
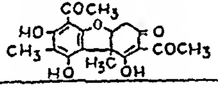
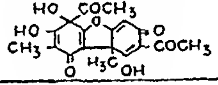
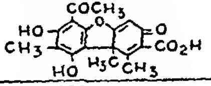
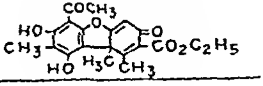
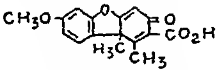
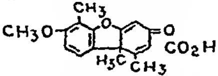
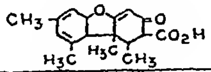
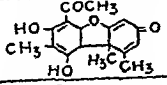
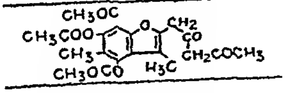
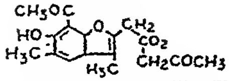
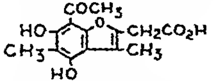
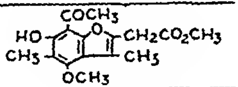
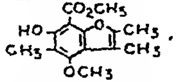
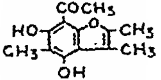
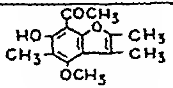
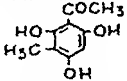
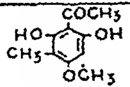
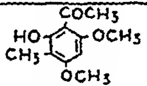
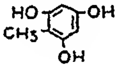
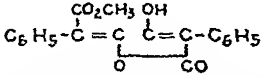
Serial No.	Substance	Structural formula where known in literature	Solubility	Growth of *M. tuberculosis* H 37 Rv — 0 means no growth — Days after inoculation	Drug conc. in μg/cc 20	10	2	0.5
0	(Control)			10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4
1	*d*-Usnic Acid		c	10	0(0)	0(2)	4(4)	4(4)
				15	0(0)	0(3)	4(4)	4(3)
				20	0(0)	0(3)	4(4)	4(3)
2	rac-Usnic Acid	’’	c	10	0	0	3	4
				15	0	0	4	4
				20	0	0	4	4
3	*d*-Diacetyl Usnic Acid		s	10	0	1	4	4
				15	0	2	4	4
				20	0	2	4	4
4‡	*d*-Monoacetyl Usnic Acid		p	10	0	0	3	4
				15	0	0	3	4
				20	0	0	3	4
5	*d*-Usnamide		p	10	2	3	4	4
				15	2	3	4	4
				20	3	3	4	4
6	*d*-N_5 Methylusnamide		p	10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4
7	*d*-Diacetyl Dihydro Usnic Acid		s	10	3	4	4	4
				15	3	4	4	4
				20	3	4	4	4
8‡	*l*-Dihydro Usnic Acid		s	10	0	1	4	4
				15	0	2	4	4
				20	0	3	4	4
9	*d*-Usnonic Acid		s	9	2	4	4	4
				12	2	3	4	4
				18	2	3	4	4
10‡	Usnolic Acid		s	10	3	4	4	4
				15	3	4	4	4
				20	4	4	4	4
11	Ethyl Usnolate		s	9	1	3	4	3
				12	2	2	4	4
				18	2	2	4	4
12†	6′-Methoxy-3′,3-dimethyl-2′,3′-dihydro-benzofurano (2′,3′,5,4-)-Δ$^{2:5}$ cyclohexadienone-2-carboxylic Acid		s	9	2	3	3	3
				12	2	3	3	4
				18	2	3	3	3
13†	6′Methoxy-3′,7′,3-trimethyl-2′,3′-dihydro-benzofurano (2′,3′,5,4-)-Δ$^{2:5}$ cyclohexadienone-2-carboxylic Acid		s	9	2	3	3	3
				12	2	3	3	4
				18	2	3	3	3
14†	3′,4′,6′,3-Tetramethyl-2′,3′-dihydro-benzofurano (2′,3′,5,4-)-Δ$^{2:5}$ cyclohexadienone-2-carboxylic Acid		s	9	3	3	3	3
				12	3	3	3	3
				18	3	3	3	3
15	Decarbousnol		s	10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4
16	Diacetyl decarbousnic Acid		p	10	4	4	4	4
				15	4	4	4	4
				20	4	4	4	4

TABLE I (Continued)

17	Decarbousnic Acid		p	10 15 20	3 3 3	3 4 4	4 4	4 4
18	Usnetic Acid		s	9 12 18	4(3) 4(3) 4(3)	4(4) 4(3) 4(3)	4(4) 4(3) 4(3)	4(4) 4(4) 4(4)
19†	Methylusnetate-4-methyl ether		p	9 12 18	3 2 2	3 4 3	4 4 4	4 4 4
20†	Methyl 2,3,5-trimethyl-4-methoxy-6-hydroxy cumarone-7-carboxylate		p	9 12 18	3 3 3	3 4 4	4 4 4	4 4 4
21	Usnetol		c	9 12 18	4(1) 4(2) 4(4)	4(1) 4(3) 4(4)	4(4) 4(4) 4(4)	4(4) 4(4) 4(4)
22†	Usnetol-4-methyl ether		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
23†	Methyl acetophloroglucinol		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
24†	Methyl acetophloroglucinol-4-methyl ether		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
25†	Methyl acetophloroglucinol-4,6-dimethyl ether		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
26†	Methyl phloroglucinol		s	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
27	Diacetyl usnic acid ethoxylate		s	9 12 18	3 3 3	3 3 4	4 4 4	4 4 4
28	Sodium *d*-usnate		s	10(9) 15(12) 20(18)	0(0) 0(0) 0(0)	2(0) 3(1) 3(2)	4(3) 4(4) 4(3)	4(4) 4(4) 4(4)
29	3′-Hydroxy usnanilide		p	9 12 18	0 0 0	2 3 3	4 4 4	4 4 4
30	4′-Hydroxy usnanilide		p	9 12 18	4* 3 3	2 2 3	4 4 4	4 4 4
31	4′-Dimethyl amino-usnanilide		p	9 12 18	4 4 4	4 4 4	4 4 4	4 4 4
32	N,β-Carboxyethyl usnamide		s	10 15 20	1 2 1	3 4 4	4 4 4	4 4 4
33	*l*-usnamido napthalene-5-sulfonic acid		s	10 15 20	4 4 4	4 4 4	4 4 4	4 4 4
34	Vulpinic acid		s	10 15 20	1 2 2	3 3 3	4 4 4	4 4 4

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* Suspension slightly turbid due to low solubility of drug. This may account for higher density readings.
† Samples of these were furnished by Prof. Alexander Robertson, F.R.S., to whom our grateful thanks are due.
‡ These differed in melting points from those previously recorded in literature, but gave correct analytical figures.
Solubility: s—solution, c—colloid, p—suspension.

might exhibit desirable solubility characteristics consisted in the condensation of usnic acid with substances bearing hydrophylic groupings. The compounds obtained in this connection are No. 29-33. Furthermore, a few substances possessing some of the structural features of usnic acid, No. 11-14, No. 19, No. 20, No. 22-27 have also been included for the examination of their antitubercular potentialities. A total of 33 compounds, related to usnic acid, have therefore been tested against the tubercle bacillus strain H37 Rv. in Dubos medium, using d-usnic acid for comparison. It appears desirable to report the results so far obtained in view of the recent publication of Shibata and Ukita on similar lines. They are given in Table I which also includes vulpinic acid.

We had hoped that this preliminary inquiry would reveal the minimum structural requirements of the usnic acid molecule essential for activity and the relationship, if any, between chemical constitution and antitubercular action in this group so that further planned progress could be made in the chemotherapy of mycobacterial infections. An examination of the present results, which are qualitatively in agreement with those of Stoll and his associates and Shibata and Ukita, could give rise to numerous generalities of a speculative nature. However, the relationship between structure and activity seems to be particularly obscure in this class of compounds. On the one hand, there is the utter lack of specificity of action of the stereoisomeric usnic acids unlike other optically and physiologically active natural products, and on the other, there is the apparently high degree of specificity of the *entire* usnic acid molecule for antitubercular activity. However, it is gratifying to find that some of the simple derivatives of usnic acid, the mono- and diacetyl usnic acids, dihydrousnic acid, m and p-hydroxyusnanilides, have activities equal to or comparable to that of the original substance. These compounds are now being subjected to further investigation.

Experimental. All tests were made by inoculating 0.1 ml of an 8-10-day-old culture diluted 6 x 10^3 into 5.9 ml of culture medium plus drug in 20 x 125 mm screw cap tubes which were then incubated at 37°C. Dubos liquid culture medium was used.[5,6] The culture tubes were previously selected to have the same optical density in the Coleman spectrophotometer to within 0.5%. Readings of the optical density of the culture in these tubes were then taken at 3, 10, 15, and 20 days after inoculation and compared with the growth of the control cultures grown at the same time.

All substances to be tested were dissolved in hot acetone, filtered thru a Corning U.F. filter into a small quantity of culture medium from which the acetone was then evacuated under reduced pressure. The sodium usnate was also tested by dissolving it in a minimal amount of warm (60°C) 2% Na_2CO_3 before addition to the culture medium. Both methods gave the same results. A few compounds gave clear solutions; the remainder were either colloidal or finely divided particulate suspensions as indicated in the table.

The optical density readings at 10, 15, and 20 days after inoculation were expressed as percent of the control readings and then classified into 4 groups, Group 1 being 1 to 25% of the control reading, Group 2, 25 to 50%, Group 3, 50 to 75% and Group 4, 75 to 100%. Zero indicates no growth as measured by optical density. The results are given in Table I. All tests were made in duplicate. In a few cases the tests were repeated at a later date. The values for these are indicated in parentheses.

Summary. The entire usnic acid molecule is involved in its action against the tubercle bacillus since any major alteration in the molecule markedly reduced or destroyed the activity. This is in marked contrast to the equal activity previously demonstrated for stereoisomeric usnic acids.

It is a pleasure to express our indebtedness to Professor Louis F. Fieser, in whose laboratories the chemical work was carried out, for his advice and encouragement, and also to Miss Blanche Burkhart for her assistance with the bacteriological tests.

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[5] Dubos, R., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

[6] Dubos, R., and Davis, B., *J. Exp. Med.*, 1946, **83**, 409.

16996

Site of Conversion of Tryptophan into Nicotinic Acid in Man.[*]

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L. EMMETT HOLT, JR.

From the Department of Pediatrics, New York University, and the Children's Medical Service, Bellevue Hospital, New York City.

Although direct evidence is now available for the conversion of tryptophan into nicotinic acid in the mammalian organism and of the intermediate steps concerned[1,2,3] the site of such conversion is still a matter of dispute. It is possible that different animal species behave differently in this regard, since there seem to be species differences[4-10] in the ability of tryptophan to spare nicotinic acid and in the proportion of excreted nicotinic acid derivatives. Studies to determine the site of the conversion are confined to experimental animals and have given contradictory results.[11] Ellinger and Abdel-Kader[12] compared the oral and parenteral routes of administration in rats and observed a far greater rise in N^1 methyl nicotinamide output after oral administration. The magnitude of this response was markedly reduced by chemotherapeutic agents. This led them to conclude that the intestinal synthesis was of primary importance.

An opposite conclusion was reached by Schweigert and Pearson[13,14] who administered tryptophan both orally and intraperitoneally. The promptness of the response by the latter route, even though it was smaller in magnitude led them to believe that the conversion took place primarily in the tissues. This conclusion was somewhat modified in a later report by Junqueira and Schweigert[15] who studied the effect of succinyl sulfathiazole on the response to oral tryptophan with and without a pteroylglutamic acid supplement. In the presence of the sulfa drug when unsupplemented by pteroylglutamic acid, oral tryptophan had very little augmenting effect on N^1methyl nicotinamide excretion; but when pteroylglutamic acid was added the urinary excretion of the N^1methyl compound rose sharply.

In order to throw further light on this question we undertook to make comparisons of the effect of oral and intravenous tryptophan administration in infants.

Experimental. The subjects were 6 normal male infants, ranging in age from 3 to 24 months and in weight from 4.1 to 11.4 kg. They were maintained on standard formulae of evaporated milk and dextrimaltose through-might exhibit desirable solubility characteristics consisted in the condensation of usnic acid with substances bearing hydrophylic groupings. The compounds obtained in this connection are No. 29-33. Furthermore, a few substances possessing some of the structural features of usnic acid, No. 11-14, No. 19, No. 20, No. 22-27 have also been included for the examination of their antitubercular potentialities. A total of 33 compounds, related to usnic acid, have therefore been tested against the tubercle bacillus strain H37 Rv. in Dubos medium, using d-usnic acid for comparison. It appears desirable to report the results so far obtained in view of the recent publication of Shibata and Ukita on similar lines. They are given in Table I which also includes vulpinic acid.

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[*] This study was supported in part by grants from the Dazian Foundation for Medical Research, the Sugar Research Foundation, and Roche Anniversary Foundation.

[†] Mead Johnson Fellow in Pediatrics.

[1] Albert, P. W., Scheer, B. T., and Deuel, H. J., Jr., *J. Biol. Chem.*, 1948, **175**, 479.

[2] Heidelberger, C., Gullberg, M. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, 1948, **175**, 471.

[3] Heidelberger, C., Abraham, E. P., and Lepkovsky, S., *J. Biol. Chem.*, 1948, **176**, 1463.

[4] Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *Fed. Proc.*, 1946, **5**, 154.

[5] Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573.

[6] Krehl, W. A., Sarma, P. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **162**, 403.

[7] Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1947, **171**, 203.

[8] Briggs, G. M., *J. Biol. Chem.*, 1945, **161**, 749.

[9] Luecke, R. W., McMillen, W. N., Thorp, F., Jr., and Tull, C., *J. Nutr.*, 1947, **33**, 251.

[10] Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

[11] Perlzweig, W. A., Rosen, F., Levitas, N., and Robinson, J., *J. Biol. Chem.*, 1947, **167**, 511.

[12] Ellinger, P., and Abdel-Kader, M. M., *Nature*, 1947, **160**, 675.

[13] Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

[14] Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1948, **172**, 485.

[15] Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, 1948, **175**, 535.

We had hoped that this preliminary inquiry would reveal the minimum structural requirements of the usnic acid molecule essential for activity and the relationship, if any, between chemical constitution and antitubercular action in this group so that further planned progress could be made in the chemotherapy of mycobacterial infections. An examination of the present results, which are qualitatively in agreement with those of Stoll and his associates and Shibata and Ukita, could give rise to numerous generalities of a speculative nature. However, the relationship between structure and activity seems to be particularly obscure in this class of compounds. On the one hand, there is the utter lack of specificity of action of the stereoisomeric usnic acids unlike other optically and physiologically active natural products, and on the other, there is the apparently high degree of specificity of the *entire* usnic acid molecule for antitubercular activity. However, it is gratifying to find that some of the simple derivatives of usnic acid, the mono- and diacetyl usnic acids, dihydrousnic acid, m and p-hydroxyusnanilides, have activities equal to or comparable to that of the original substance. These compounds are now being subjected to further investigation.

Experimental. All tests were made by inoculating 0.1 ml of an 8-10-day-old culture diluted 6 x 10^3 into 5.9 ml of culture medium plus drug in 20 x 125 mm screw cap tubes which were then incubated at 37°C. Dubos liquid culture medium was used.[5,6] The culture tubes were previously selected to have the same optical density in the Coleman spectrophotometer to within 0.5%. Readings of the optical density of the culture in these tubes were then taken at 3, 10, 15, and 20 days after inoculation and compared with the growth of the control cultures grown at the same time.

All substances to be tested were dissolved in hot acetone, filtered thru a Corning U.F. filter into a small quantity of culture medium from which the acetone was then evacuated under reduced pressure. The sodium usnate was also tested by dissolving it in a minimal amount of warm (60°C) 2% Na_2CO_3 before addition to the culture medium. Both methods gave the same results. A few compounds gave clear solutions; the remainder were either colloidal or finely divided particulate suspensions as indicated in the table.

The optical density readings at 10, 15, and 20 days after inoculation were expressed as percent of the control readings and then classified into 4 groups, Group 1 being 1 to 25% of the control reading, Group 2, 25 to 50%, Group 3, 50 to 75% and Group 4, 75 to 100%. Zero indicates no growth as measured by optical density. The results are given in Table I. All tests were made in duplicate. In a few cases the tests were repeated at a later date. The values for these are indicated in parentheses.

Summary. The entire usnic acid molecule is involved in its action against the tubercle bacillus since any major alteration in the molecule markedly reduced or destroyed the activity. This is in marked contrast to the equal activity previously demonstrated for stereoisomeric usnic acids.

It is a pleasure to express our indebtedness to Professor Louis F. Fieser, in whose laboratories the chemical work was carried out, for his advice and encouragement, and also to Miss Blanche Burkhart for her assistance with the bacteriological tests.

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[5] Dubos, R., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

[6] Dubos, R., and Davis, B., *J. Exp. Med.*, 1946, **83**, 409.

tinamide excretion produced by this feeding was in the same range as that which resulted from the intravenous administration. Differences between the two routes of administration are within the limits of the usual daily variations that occur in the excretion of this substance. We have noted that the daily N^1 methyl nicotinamide excretion may fluctuate as much as 1.7 mg even when the infant is maintained on a constant diet.

Discussion. The rise of urinary N^1methyl nicotinamide excretion produced by the intravenous administration of L-tryptophan leads to the conclusion that the conversion of tryptophan to nicotinic acid in man takes place in the tissues rather than in the gastrointestinal tract as a result of bacterial action. This premise is strengthened both by the fact that the N^1methyl nicotinamide values are the same regardless of the route of administration, and by the fact that this increase takes place so promptly. For this conversion to occur as a result of tryptophan excretion into the gastrointestinal tract and subsequent action by bacteria there, one would anticipate a considerable time lag which actually did not occur.

The differences in the rates of administration are sufficient to explain the variance of our results from those of Schweigert *et al.*[13-15] One can postulate that our slow rate of injection raised the blood tryptophan level for a prolonged period of time thereby giving the tissues more time to produce the conversion. The method of a single large injection may provide such a large excess of tryptophan that it may be disposed of in other ways. This may also account for the results obtained by Ellinger and Abdel-Kader[12] who do not give any details regarding the technic of their parenteral tryptophan.

Of interest in this connection are the recent communications of Schweigert *et al.*[17] and of Singal, Sydenstricker and Littlejohn.[18] The former, working with chick embryos observed an increase in the nicotinic acid content of the tissues after an injection of tryptophan. The latter authors reported that the administration of L-tryptophan to rats caused an increase above normal of the nicotinamide concentration of the liver, a phenomenon observed in this organ alone.

Summary. The intravenous administration of one gram of L-tryptophan to infants on constant diets gave a prompt and large rise in the urinary N^1methyl nicotinamide which was equal in magnitude to that provoked by oral administration of the tryptophan. In view of these findings, the conversion of tryptophan to N^1methyl nicotinamide in man seems to be mediated by the body tissues rather than by bacterial synthesis in the gastrointestinal tract.

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[17] Schweigert, B. S., German, H. C., and Garber, M. J., *J. Biol. Chem.*, 1948, **174**, 383.

[18] Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1948, **176**, 1069.

16997

Effect of Iodide on Thyroid Glands of Rats Kept at Low Temperature.

ARTHUR J. LESSER, RICHARD J. WINZLER, AND J. B. MICHAELSON.
From the Departments of Pharmacology and Biochemistry, University of Southern California School of Medicine, Los Angeles, Calif.

A number of workers have noted that the thyroid glands of rats kept in the cold develop hyperplasia, thickened acinar epithelium, and loss of colloid characteristic of a hyperactive thyroid gland. It was of interest to determine whether the administration of iodide to rats kept under cold-room conditions would cause involution of the thyroid gland as it does in toxic goiter.

Four groups of male albino rats of the

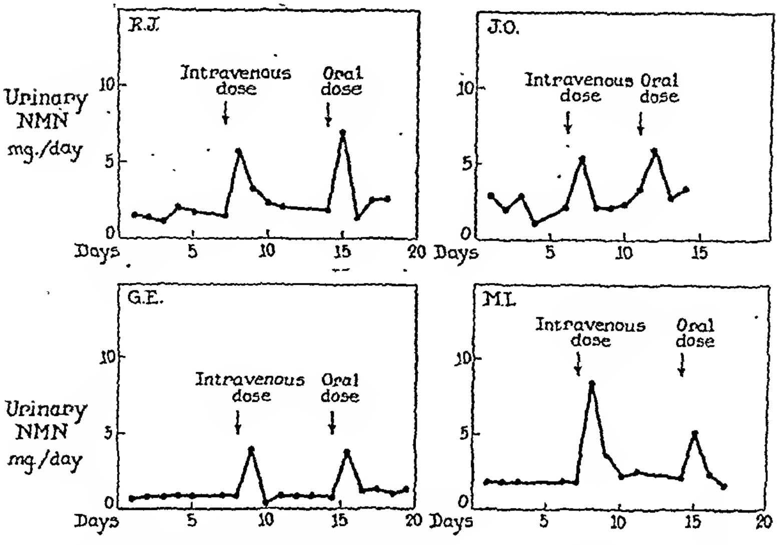

FIG. 1.
Effect of an intravenous and an oral dose of *L-tryptophane* on the urinary N^1methyl nicotinamide excretion.

out the period of study. After the urinary excretion of N^1methyl nicotinamide became fairly stable, the test dose of one gram L-tryptophan was administered either intravenously or orally.

The tryptophan solution for intravenous use was prepared in the following manner. One gram was dissolved in 30 cc of sterile distilled water containing 5 cc of 6 N hydrochloric acid, water was added to make a volume of 300 cc, and then the pH was adjusted with sodium hydroxide to neutrality. The solution was passed through a Seitz filter and then autoclaved for 10 minutes at 15 lb pressure. It was administered by slow drip, over a period of 3 to 6 hours. In 4 of the subjects the tryptophan was also administered orally, one week after the intravenous injection. The oral dose was divided in half and fed with the 10 A.M. and 2 P.M. bottles.

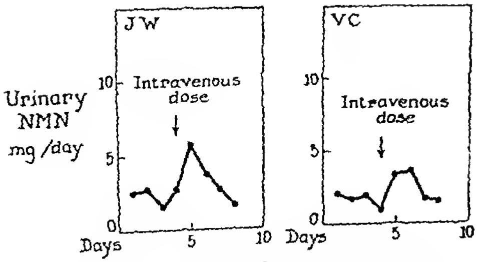

FIG. 2.
Effect of an intravenous dose of *L-tryptophane* on the urinary N^1methyl nicotinamide excretion.

The N^1methyl nicotinamide determinations were performed according to the method of Huff and Perlzweig[16] on 24 hour collections of urine. The urines were collected in amber bottles with enough glacial acetic acid to make a 2% solution.

Results. The data for the 6 subjects are expressed graphically in Figs. 1 and 2. The slow intravenous administration of one gram of L-tryptophan resulted in a marked increase of urinary N^1methyl nicotinamide on the day of the infusion in 5 of these 6 subjects. In the sixth subject (V.C., Fig. 2), there was a moderate increase both on the day of as well as the day following the tryptophan injection. This slighter response can perhaps be attributed to the much larger size of this particular infant who received the same dose as the smaller subjects. We do not have an explanation for the delay in excretion.

In those subjects who also received the tryptophan orally, the rise in N^1methyl nico-

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[16] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, 167, 157.

TABLE I.
Effect of Iodide on the Thyroid Glands of Rats at Room Temperature or at 4°C.

Group	Treatment	No. of animals*	Wt gain	Thyroid weight, mg/100 g	Epithelial height, microns	Colloid diameter, microns§
A	Room temp. stock diet	5	10.8	10.4	9.1	41
B	Room temp. stock diet 0.05% KI	4	—26.3	12.7	3.5	60
C	4°C stock diet	11†	—18	12.8	12.9	34
D	4°C stock diet 0.05% KI	5‡	—43	13.4	8.3	47

* No. of experimental animals varies due to deaths during the course of the experiment. Figures represent animals surviving at end of 21 days.

† Including 4 animals killed at end of 2 weeks.

‡ Including 2 animals killed at end of 2 weeks.

§ Mean of long and short diameters.

develops like a salivary gland which later loses its excretory pathways, leaving the thyroglossal duct as a remnant. Colloid may, therefore, be considered as a secretory product of the thyroid gland trapped in the alveoli. Iodide is known to cause hypersecretion and hypersalivation and may therefore also be considered to induce secretion of colloid into the thyroid follicles. Such colloid would, from the results of Wolff and Chaikoff, be expected to be low in organic iodine.

Summary. The hyperplasia of the thyroid epithelium produced in rats by exposure to cold-room conditions for 3 weeks can be prevented by increasing the iodide level of the diet. The mechanism of this effect would appear to correspond to the involuting action of iodide in the thyroids of patients with hyperthyroidism. It is difficult to explain these effects on the basis of the inhibition of hormone synthesis, and it is submitted that iodide may stimulate the secretion of colloid into the lumen of thyroid follicles by a mechanism related to its stimulating influence on salivation.

16998

Tetanus Prophylaxis with Penicillin-Procaine G.

Milan Novak, Milton Goldin, and Welton I. Taylor.
From the Department of Bacteriology, College of Medicine, University of Illinois, Chicago.

Clostridium tetani is sensitive to penicillin *in vitro*[1] and *in vivo*.[2,3] The development of penicillin-procaine has resulted in prolonged therapeutic blood levels following a single injection.[4,5] Claims have been made for inhibitory levels which are maintained for as

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[1] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, E. D., Heatley, N. G., Jennings, M. A., Florey, H. W., *Lancet*, 1941, **2**, 177.

[2] Weinstein, L., and Wesselhoeft, C., *New England J. Med.*, 1946, **233**, 681.

[3] Diaz-Rivera, R. S., Deliz, L. R., Berio-Suarez, J., *J.A.M.A.*, 1948, **138**, 191.

[4] Herrell, W. E., Nichols, D. R., and Heilman, F. R., *Proc. Staff Meet., Mayo Clinic*, 1947, **22**, 567.

[5] Sullivan, N. P., Symmes, A. T., Miller, H. C., Rhodehamel, H. W., Jr., *Science*, 1948, **107**, 169.

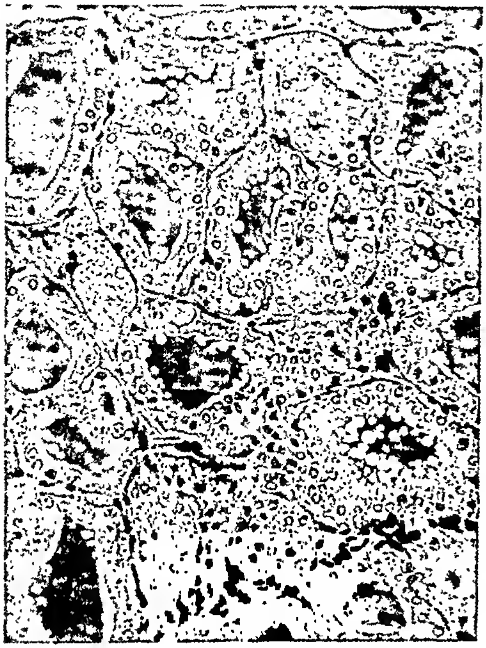

FIG. 1.

University of Southern California strain weighing between 200 and 350 g were used. Groups A and B were kept in single cages at room temperature (21 to 25°C) and groups C and D were kept in single cages with wire bottoms in a cold room maintained at 4°C. Groups A and C were on a stock diet consisting of ground oats 33%, cottonseed oil fortified with vitamins A and D 10%, wheat flour 38%, skimmed milk 14%, alfalfa 4%, yeast 2%, NaCl 0.5%, and $CaCO_3$ 0.5%. Groups B and D were maintained on this same diet to which had been added 50 mg KI per 100 g diet. The food intake and weight changes were determined at 3-day intervals.

At the end of 3 weeks, the animals were weighed, sacrificed, and the thyroid glands removed and weighed. The glands were fixed in formalin and stained with hematoxylin-eosin for histological examination. (In 6 cases animals were killed at the end of 2 weeks). The average epithelial heights and colloid diameters were determined on 100 different follicles from different parts of the thyroid glands.

Fig. I shows representative histological sections from thyroid glands of animals from the 4 groups. IA is typical of thyroid sections from Group A and shows a histological picture characteristic of a normal, moderately-active gland. 1B is a section from the thyroid gland of a rat from Group B receiving iodide at room temperature. It is seen that the gland is less active with thinner epithelium and more colloid. Exposure to low temperatures for 3 weeks brings about a marked hyperplasia with loss of colloid and thickened epithelium (1C). Administration of iodide prevented this hyperplasia and brought about a more normal-appearing thyroid gland in spite of the exposure to cold (1D).

Table I shows the data on the thyroid weights, epithelial heights, and colloid diameters of the 4 groups.

The data in Table I amplify the conclusions illustrated by Fig. 1 and show that iodide greatly reduces the thickness of the thyroid acinar epithelium and increases the colloid in the glands of animals made hyperactive by exposure to cold. These results are in accord with those obtained by Starr and Roskelley,[1] and stimulates the involuting effect of iodine in hyperthyroidism. It seems likely that iodine reduces the activity of the thyroid gland by a similar mechanism in both cases. It is of interest that the weight of the thyroid gland varied but little in the various groups—the amount of epithelium being inversely related to the amount of colloid.

Little can be said as to the mechanism of this involuting action of iodide at the present time. The work of Wolff and Chaikoff[2] clearly shows that increases in the blood iodide levels inhibit the formation of thyroxine by the thyroid gland of rats. Such an observation does not, however, explain the fact that iodide causes an accumulation of colloid in the thyroid follicles of cold-treated rats or of hyperthyroid individuals. It is possible that the effect of iodide on colloid accumulation may be brought about by a mechanism not related to hormone synthesis. The thyroid

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[1] Starr, P., and Roskelley, R., *Am. J. Physiol.*, 1940, **130**, 549.

[2] Wolff, J., and Chaikoff, I. L., *J. Biol. Chem.*, 1948, **174**, 555.

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[4] Herrell, W. E., Nichols, D. R., and Heilman, F. R., *Proc. Staff Meet., Mayo Clinic*, 1947, **22**, 567.

[5] Sullivan, N. P., Symmes, A. T., Miller, H. C., Rhodehamel, H. W., Jr., *Science*, 1948, **107**, 169.

TABLE I.
Mortality in Penicillin-Treated Mice Infected with *Cl. tetani*.

Series	Units of penicillin	Hours before penicillin	Total animals	Survivors	Time to death (days)	Mortality, %
A	0	0	32	1	<4	96.8
B	150	0	25	20	7-10	20.0
C	150	0	25	5	<5	80.0*
D	300	0	25	21	4- 8	16.0
E	150	3	25	21	7-10	16.0
F	150	6	18	14	6-10	22.2
G	150	24	19	9	<5	52.6

* *Penicillin injected IM in opposite leg rather than infected leg.*

long as 4 or 5 days following injection of 300,000 or 600,000 units.

The possibility of penicillin action against spores of tetanus in tissue during the incubation period of the disease posed the probability of its use as a prophylactic agent. Its value in this instance would be especially desirable in minor injuries where there is hesitancy in the use of antitoxin because of the dangers from foreign protein sensitization. Its use to prevent toxin formation in tissue would seem more rational than use of antitoxin to neutralize toxin after it is formed.

The results obtained with mice as shown in data presented demonstrate a definite prophylactic effect. More detailed evidence will be presented later.

*Methods. Clostridium tetani** was grown in Trypticase Soy Broth with added fresh calf brain at 37°C for about 12 days, then filtered through sterile cheesecloth to remove meat particles, centrifuged and resuspended in sterile distilled water in 8 oz. screw-capped bottles. Toxin and vegetative cells were destroyed by heating in a water bath for 30 minutes at 80°C. Spore suspensions were then shaken vigorously 30 times to suspend spores evenly, and counted by adding serial dilutions in duplicate to melted and cooled Trypticase Soy Broth (1.5% agar added) deeps. After solidification a one-half inch layer of 2% stratifying agar was poured on top to insure adequate anaerobiosis. The appearance of macroscopic colonies upon incubation yielded a count only of viable spores. Spore suspensions were then adjusted to 1,000,000/ml. and stored at 4°C.

Since it has been adequately demonstrated that the spores of *Cl. tetani* are not infective in healthy tissue, $CaCl_2$ in 5% solution was used as a tissue debilitant.[6-8]

The number of spores necessary to produce approximately 100% mortality in White Swiss mice was determined by injecting mice with 0.1 ml 5% $CaCl_2$ immediately followed by 10, 100, 1,000, 10,000, and 100,000 spores of *Cl. tetani* intramuscularly in the inner surface of the left hind leg. Mice receiving 10 and 100 spores survived; those receiving 1,000 spores died with symptoms of tetanus in 3-5 days, and with 10,000 and 100,000 spores, 2-3 days. Thus 1,000 spores was chosen as an LD_{100} infective dose.

To determine the possible prophylactic value of penicillin in tetanus, Penicillin G Procaine in oil with 2% aluminum monostearate,† containing 300,000 units/ml was used. The contents of one disposable cartridge containing 300,000 units were added to 99 ml sterile sesame oil and shaken 30 times before each usage to procure a homogenous suspension. With this dilution 0.1 ml contained 300 units penicillin, 0.05 ml contained 150 units. (The latter is comparable to 600,000 units for an adult human being.) Injections of penicillin were made in the same leg used for the infective dose of spores and $CaCl_2$, except in one series (Table I, Series C) where the opposite leg was used.

The time lapse between the injections of

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* Courtesy of Parke Davis and Company, Detroit, Mich.

[6] Bullock, W. E., and Cramer, W., *Proc. Royal Soc. London*, 1919, B, **90**, 513.

[7] Russell, D. S., *Brit. J. Exp. Path.*, 1927, **8**, 377.

[8] Fildes, P., *Brit. J. Exp. Path.*, 1927, **8**, 387.

† Supplied by Abbott Laboratories, North Chicago, Ill.

TABLE II.
Statistical Analysis of Data on Mortality and Time to Death.

	Mortality		Time to death		
			Days		
Series	%	P value*†	Mean	S.D.	P value*‡
A	96.8	—	3.31	1.42	—
B	20.0	<.01	8.40	3.47	<.01
C	80.0	>.10	3.90	1.69	>.05
D	16.0	<.01	6.25	2.51	<.01
E	16.0	<.01	7.75	2.94	<.01
F	22.2	<.01	6.75	3.07	<.01
G	52.6	<.01	4.10	2.11	>.05

* Series compared with Series A.

† P values taken from Chi-square 4-fold table including Yates correction; value less than 0.05 indicates significant difference.

‡ P values taken from Fisher's "t" table; value less than 0.05 significant.

S.D., Standard Deviation.

the infective dose and penicillin was varied, since other reports on the clostridia[9-15] indicate that elapsed time is an important factor in protection.

White Swiss mice of 15 to 35 g weight were used with a holding period of 10 days after the initial infective dose. Results after this interval are shown in Table I.

A statistical analysis of data[16] on mortality and time to death of mice receiving penicillin prophylaxis and untreated controls is presented in Table II.

The P value (likelihood of difference arising through chance alone) of the mortality of each series as compared with controls (Table II, Series A) was obtained from a Chi-square test of independence 4-fold table including Yates correction. P values of series B, D, E, F, and G (Table II) are significant.

The statistical analysis of the difference in time to death in days between each series and series A includes the mean time to death in days and the standard deviation with the P value obtained from Fisher's table indicating the significance of the data. Series B, D, E, and F have significant values.

Discussion. Both the reduction in mortality and prolongation of life of treated mice indicate the prophylactic effect of the penicillin-procaine compound. Administration of penicillin within 24 hours after the injection of spores of *Cl. tetani* serves to reduce the mortality as compared with the untreated controls. The lowest mortalities resulted when 150 and 300 units of penicillin were given in the infected leg immediately, and when 150 units were given after 3 and 6 hours' delay (Table I, Series B, D, E, and F). These series having low mortalities were also observed to have the greatest time lapse before symptoms and deaths. When compared to the rapid completion of the disease as observed in the controls (Table I, Series A), the noticeably longer time required before symptoms and deaths occur in the low mortality series receiving penicillin (Table I, Series B, D, E, and F) may indicate that the number of organisms capable of producing toxin is considerably reduced and that only when there are organisms surviving in remaining necrotic areas, which multiply and produce a lethal dose of toxin, does a fatality result. The similarity between the mortality rates

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[9] Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G., *Lancet*, 1940, 2, 226.

[10] McIntosh, J., and Selbie, F. R., *Lancet*, 1942, 2, 750.

[11] Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *Ann. Int. Med.*, 1943, 19, 707.

[12] McKee, C. M., Hamre, D. M., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1943, 54, 211.

[13] McIntosh, J., and Selbie, F. R., *Lancet*, 1943, 2, 224.

[14] Hac, L. R., and Hubert A. C., PROC. SOC. EXP. BIOL. AND MED., 1943, 53, 61.

[15] Hac, L. R., *J. Inf. Dis.*, 1944, 74, 164.

[16] Snedecor, G. W., *Statistical Methods*, Ames, Iowa, Iowa State College Press, 1946, 485 pp.

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TABLE I.
Observations on Rh Sensitization in Males.

No. of inj.	No. of subjects	Frequency of Rh antibody formation		% of total series first showing Rh antibodies at inj. in question	Cumulative total (%)	
		No.	%		Sensitized	Not sensitized
2	47	18*	38.3	38.3	38.3	61.7
3	23	6	26.1	26.1 × 0.617 = 16.1	54.4	45.6
4	15	3	20.0	20.0 × 0.456 = 9.1	63.5	36.5
5	10	1	10.0	10.0 × 0.365 = 3.7	67.2	32.8
6	3	1	33.3	33.3 × 0.328 = 10.9	78.1	21.9

* *One subject produced Rh antibodies after the very first injection. In this case a history was elicited of several blood transfusions received many years previously during treatment for osteomyelitis.*

O, Rh_0-positive blood, each dose consisting of 2 to 4 cc of a 50% suspension of whole blood in sodium citrate solution. The injections were given at intervals of 3 to 4 months. All the injections were given intravenously, except that occasionally part of the blood was deposited into or under the skin. Tests for Rh antibodies were made in the usual manner[5,6] by the agglutination and albumin-plasma conglutination methods. The antibody tests were carried out 10 to 14 days after each injection, except that in most cases no tests were done after the very first injection. According to our previous observations,[2,4] as a rule a minimum of 2 injections, spaced 3 to 4 months apart, are needed to induce the formation of Rh antibodies, the first injection acting as a primer and the second or subsequent injection stimulating the production of specific antibodies.

The results are summarized in Table I. It will be seen that after the second injection, 18 of the 47 subjects, or 38.3%, had demonstrable antibodies in their sera. One of these 18 subjects had showed antibodies after the very first injection, but after careful questioning a history was elicited of previous blood transfusions many years before when this individual had been treated for osteomyelitis. Of the 29 subjects who failed to respond to the second injection, 23 returned for a third injection, and 6 of these or 26.1% then showed antibodies. If we assume that the 6 individuals who did not return for the third injection had an equal chance of becoming sensitized, then this indicates that the third injection would sensitize 26.1% of the 61.7% of the nonsensitized individuals remaining after the second injection, or 16.1% of the total number injected. By adding this 16.1% to the 38.3% representing the individuals sensitized by the second injection, we find that fully 54.4% of the Rh-negative individuals became sensitized after a course of 3 properly spaced injections of Rh-positive blood. Of the 17 individuals who failed to respond to the third injection, 15 returned for a fourth injection, and of these 3 individuals or 20% became sensitized. As shown in the table, this raises to 63.5% the total incidence of sensitization after a course of 4 injections; and similarly, the percentage of sensitized individuals is raised to 67.2% by a fifth injection of Rh-positive blood. Unfortunately, only 3 subjects returned for the sixth injection, of whom one became sensitized. Obviously, this result has a large statistical error and should therefore be discounted. Judging from the results of the third, fourth, and fifth injections, it would appear as if the percentage of sensitized individuals was approaching a limit somewhere between 70 to 80%; while if the results of the sixth injection could be taken literally, it would seem that all the injected individuals would eventually become sensitized following a sufficient number of injections of blood. This question can obviously only be settled by experiments on a larger series of individuals, over a longer course of injections.

In a parallel series of experiments, a number of type Rh_1Rh_1 individuals was subjected

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[5] Wiener, A. S., *Am. J. Clin. Path.*, 1946, **16**, 477.

[6] Wiener, A. S., and Hurst, J. G., *Exp. Med. and Surg.*, 1947, **5**, 285.

and time lapse before death in series B, where an immediate injection of penicillin was given, and in series E and F where administration of penicillin was delayed for 3 and 6 hours respectively, may be explained by the fact that it is generally acknowledged that penicillin is most effective against sensitive microorganisms during the period of active growth and multiplication; therefore the efficacy of penicillin administered after 3- or 6-hour lapses may indicate that those times allow germination of spores[8] and the vegetative cells are then inhibited by the concentration of penicillin available. Since Penicillin G Procaine in oil is reported to maintain concentrations at effective therapeutic levels for 96 hours, the longer time lapse before symptoms appear may be accounted for if it is only after this level has dropped that a lethal toxin is produced by survivors.

Higher mortalities were observed in series C and G. When 150 units of penicillin was administered with no delay but in the leg opposite the necrosis, 80% of the mice died (Table I, Series C). When the penicillin was injected into the necrotic leg after a 24-hour delay, 52.6% of the mice died (Table I, series G). The indication in series C is that (1) a sufficient amount of penicillin does not reach the necrotic area to inhibit growth and production of toxin by *Cl. tetani*, with resultant death of the animal, or (2) that organisms produce a lethal dose of toxin before being inhibited, with this latter possibility being especially applicable to series G. The rate at which penicillin is released and the rate and extent of penetration into necrotic areas are probably the significant factors. The similarity of series C and G to series A (Table I) in the short time lapse before death indicates that toxin production had taken place rapidly, and if series G is compared with series F this observation is further verified since the additional 18 hours time lapse before penicillin was administered caused a 30% increase in mortality.

Conclusions. Results indicate that penicillin-procaine G in sesame oil and 2% aluminum monostearate is of significant value prophylactically in lowering the mortality of mice experimentally infected with a lethal dose of detoxified spores of *Clostridium tetani.* In addition to decreasing the mortality, it retards the development of symptoms and resulting deaths. Injected into the necrotic areas, it is more effective than the same unitage injected at an uninfected site. Whether this is caused by insufficient penetration of the drug due to the presence of necrotic tissue or to interference by the calcium chloride has not been determined.

16999

Further Observations on Isosensitization to the Rh Factor.

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From the Serological Laboratory, Office of the Chief Medical Examiner, New York City.

The purpose of this report is to describe the results of experiments on Rh sensitization in man. The findings are of significance in relation to the pathogenesis of intragroup hemolytic transfusion reactions,[1,2] and the pathogenesis of erythroblastosis fetalis,[3] as well as the practical problem of producing Rh testing sera.[4]

The subjects used in these experiments were 47 adult male individuals. Each individual was subjected to a series of injections of group

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[1] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

[2] Unger, L. J., and Wiener, A. S., *Am. J. Clin. Path.*, 1945, **15**, 280.

[3] Wiener, A. S., *Am. J. Dis. Child.*, 1946, **71**, 14.

[4] Wiener, A. S., and Gordon, E. B. S., *Am. J. Clin. Path.*, 1947, **17**, 67.

is also clear that an integrated picture of the malaria problem cannot be had until our knowledge includes as much about the non-human species of malaria as it does of the plasmodia of man.

For these reasons, the authors attempted to adapt the Ball culture method to *Plasmodium gallinaceum*, the parasite of chicken malaria, but with only partial success, and it then seemed evident that some careful studies of its biochemistry would be necessary before greater success could be hoped for. Since the addition of more glucose to the medium than originally called for seemed to improve its performance somewhat, a detailed and quantitative study of glycolysis in this species was undertaken.[¶]

Materials and Methods. The strain of *Plasmodium gallinaceum* used was obtained from Dr. Gilbert Otto of the Johns Hopkins University School of Hygiene and Public Health. Inoculation of the chicks was done either intraperitoneally or intramuscularly with brain suspensions or parasitized blood, usually when they were about three weeks old. Blood for experimental use was obtained by intracardial puncture, without anesthesia, to insure normal blood sugar levels. To avoid possible effects of a developing immunity, chicks were used during the period of rising parasitemia. The anticoagulant was Lederle heparin.

After removal from the bird the blood was taken immediately to the constant temperature (40°C) room, 0.5 ml was saved for immediate examination, and the balance placed in a rocker dilution tube, equilibrated with 5% CO_2 and 95% air, and rocked according to the Ball cultivation technic, samples being taken at regular intervals for glucose determinations, which were always made in duplicate. These were plotted against time and the hourly rate of glucose consumption calculated. This last was done per ml of blood, both normal and parasitized, and per mature red cell, reticulocyte, and parasite.

Glucose levels were determined by the Folin-Malmros method (Klett-Summerson Clinical Manual), except that a ferric duponal solution[4] was substituted for the ferric gum-ghatti solution originally specified, thus avoiding the partial precipitation of the Prussian blue which sometimes occurred with the gum ghatti. The glucose values usually differed from their mean by no more than ± 3 mg %. Erythrocyte and parasite counts were made on each sample, enough parasites being counted to keep the error within 10%[5] Differentials were also made. The stain used was the J.S.B.[6]

Whole undiluted blood was employed for all determinations. For the study of the glucose consumption of reticulocytes, chicks were made anemic by 3 previous daily injections of phenylhydrazine hydrochloride.

Results. The hourly glucose consumption rates of normal chicken blood and of blood containing a high proportion of reticulocytes (79.5%) are illustrated in Fig. 1 and 2 respectively. Fig. 3 shows in similar fashion the hourly glucose consumption of parasitized blood. With larger numbers of parasites, or greater proportions of reticulocytes, curves with even steeper initial slopes would be obtained.

Five determinations were made of the hourly glucose consumption of unparasitized

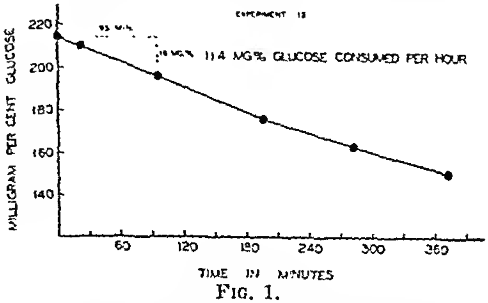

FIG. 1.

Glucose consumption of unparasitized blood containing approximately 100% adult red cells.

[¶] Since the writing of this report, the paper by Marshall (*Brit. J. Pharm.*, 3, 1) has come to our attention. Although his determinations of oxygen uptake of parasitized blood indicate glycolysis values for *P. gallinaceum* essentially like ours, added glucose failed to stimulate respiratory activity.

[4] Klenshoj, N. C., and Hubbard, R. S., *J. Lab. and Clin. Med.*, 1939, 25, 1102.

[5] Hartman, E., *Am. J. Hyg.*, 1927, 7, 407.

[6] Singh, J., and Bhattacharji, L. M., *Ind. Med. Gaz.*, 1944, 79, 102.

to a series of injections of type rh blood in an attempt to induce sensitization to the factor hr' or Hr_0, or both. Nineteen of these individuals received 2 injections; of these 13 received a third injection, 8 received a fourth injection, 5 received a fifth injection, while 4 received a sixth injection. Not one of the individuals of this series showed any Hr antibodies at any time. This, despite the smaller number of individuals injected, is in sharp contrast to our experiences with sensitization against Rh_0 factor. Moreover, these results conform with the conclusions based on clinical observations that the Hr factors are much weaker antigens than the Rh factors.[7]

Summary. The results of experiments on sensitization of male type rh individuals by intravenous injections of Rh_0-positive blood at intervals of 3 to 4 months are described. Of 47 subjects receiving 2 injections, 38.3% became sensitized; after 3 injections, the incidence of sensitization rose to 54.4%; after 4 injections, the percentage rose to 63.5%, while after 5 injections the incidence of sensitization was 67.2%. Because of the small number of individuals receiving more than 5 injections, it is not possible to state with certainty whether the incidence of sensitization approaches a maximum limit somewhere between 70 and 80% of individuals injected, or whether all individuals eventually would become sensitized providing they are given a sufficient number of injections. In any event, it is obvious that the deliberate injection of Rh-positive blood is a potent means of sensitizing Rh-negative individuals.

In contrast to these results, not one among 19 type Rh_1Rh_1 individuals receiving repeated injections of type rh blood became sensitized to the Hr factors. These results confirm the conclusion based on clinical observations that the Hr factors are much weaker antigens than the Rh factors.

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[7] Wiener, A. S., *J. Lab. and Clin. Med.*, 1948, **33**, 985.

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Glycolysis in *Plasmodium gallinaceum*.*

REGINALD D. MANWELL AND PHILIP FEIGELSON.†
From the Department of Zoology, College of Liberal Arts, Syracuse University.

For many years a practical method of cultivating the malaria plasmodia has been sought, and recently one has been devised by Ball and his colleagues[1,2] for the erythrocytic stages of *Plasmodium knowlesi,* a parasite of monkeys. Monkeys, however, are expensive and much less easily maintained in the laboratory than birds, and for this reason it is important that a good culture technic be devised for the avian plasmodia,‡ which are equally suited for the screening of antimalarial drugs and for other types of research in malaria. It

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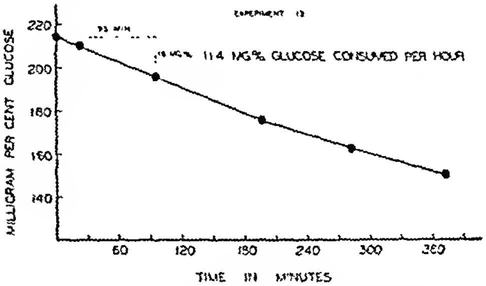

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TABLE I.
Summary of Experimental Data.*

A. Normal Unparasitized Blood.

Experiment	E	A	R	Mg glucose per hr per ml blood		X
13 (Fig. 1)	1.91	1.000	0.000	0.114		5.97×10^{-2}
14	1.86	0.995	0.005	0.064		3.36×10^{-2}
15	2.25	1.000	0.000	0.083		3.68×10^{-2}
					Mean:	4.34×10^{-2}

B. Anemic Unparasitized Blood.

Experiment	E	A	R	Mg glucose per hr per ml blood		X'
21 (Fig. 2)	0.67	0.205	0.795	0.161		2.91×10^{-1}
22	1.58	0.335	0.665	0.360		3.21×10^{-1}
					Mean:	3.06×10^{-1}

C. Parasitized Blood.

Experiment	E	A	R	P	a	b	c	Mg glucose per hr per ml blood		Mg glucose per hr per 10^7 μ^2 par. area
11 (Fig. 3)	1.33	0.895	0.105	178	59	36	5	0.54		2.57×10^{-4}
12	1.81	0.990	0.010	61	15	76	9	0.42		2.27×10^{-4}
16	0.78	0.650	0.350	200	4	93	3	0.75		3.24×10^{-4}
18	1.87	0.810	0.190	10.5	18.5	16.7	64.8	0.35		2.66×10^{-4}
19	1.34	0.562	0.438	19.8	0.0	98	2	0.36		4.27×10^{-4}
									Mean:	3.00×10^{-4}

* Abbreviations used in Table I, and in the formulae:

a, b, c = percentage distribution of 3 parasite types

$$E = \frac{\text{erythrocytes per mm}^3}{10^6} \qquad R = \frac{\text{reticulocytes}}{\text{total red count}}$$

X = mg glucose per hr per 10^9 red cells

$$A = \frac{\text{adult erythrocytes}}{\text{total red cell count}}$$

P = parasites per 100 red cells

X' = mg glucose per hr per 10^9 reticulocytes

TABLE II.
Glucose Consumption (mg per hour).

Authority	Erythrocytes (per red cell)	Erythrocytes (per μ^2 of area)	Parasites (per μ^2 area)	Ratio (col. 2/col. 3)
Our work	4.34×10^{-11}	3.42×10^{-13}	300×10^{-13}	1/87.8
Silverman *et al.*	6.25×10^{-12}	4.92×10^{-14}	390×10^{-14}	1/79.0
Ratio (our values to Silverman's)	6.95/1		7.7/1	

mg per hour for normal erythrocytes (adult), and 300×10^{-13} for parasites. Both values greatly exceed those of Silverman and colleagues (Table II), and we believe the differences are probably due to the different treatment given the blood in our work, since we used no diluting agent and did not wash the cells before making the tests, thus keeping the cell environment more nearly physiologically normal.

To make these calculations, and also to predict the glucose consumption of cultures containing any given number of parasites, the following formula was developed:

$$W_t = 4.34E \times 10^{-2} \left(A + 7.05R + 6.92P \times 10^{-5} (a + 12b + 48c)\right)$$

in which

W_t = mg glucose consumed per hour per ml of parasitized blood

a, b, and c = percentage distribution of 3 parasite types

A, E, R, X, and X' are as indicated above (formula used in computing glucose consumption of unparasitized blood)

The formula is easily derived, assuming

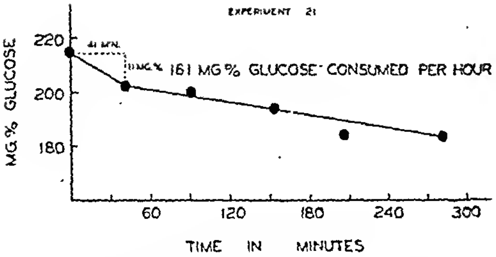

FIG. 2.

Unparasitized blood containing approximately 20.5% adult red cells and 79.5 reticulocytes.

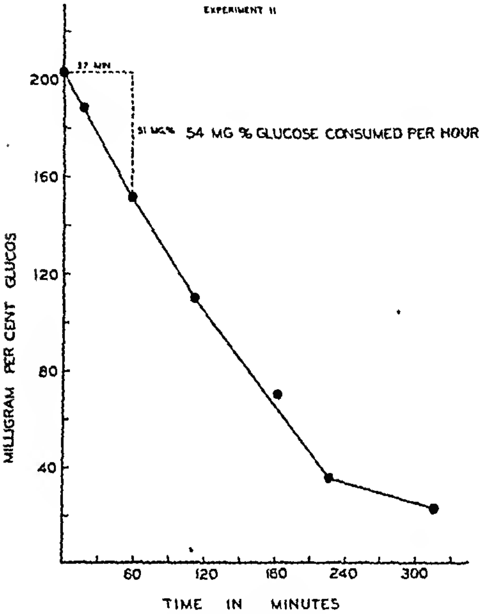

FIG. 3.

Glucose consumption of parasitized blood, containing 89.5% adult red cells, 10.5% reticulocytes, and 178 parasites per 100 red cells (Type A, 59%, Type B 36%, and Type C 5%).

blood, 3 of these on normal and 2 on highly anemic samples. The results were 0.114, 0.064, and 0.083 mg per ml on the former, and 0.161 and 0.360 mg per ml on the latter. From these figures the hourly glucose consumption per 10^9 red cells (for both mature erythrocytes and reticulocytes) was calculated.‖ The mean value for mature erythrocytes was found to be 4.34×10^{-2} mg and for reticulocytes 3.06×10^{-1}. Thus the average mature red cell has a metabolic rate only about one-seventh that of a reticulocyte.

The hourly glucose consumption rate of parasitized cells is much higher, although it depends on the size and stage in the asexual cycle of the parasite. Five determinations were made of the glucose consumption of parasitized blood. (Table I) The mean value, in mg per hour per ml, was 0.48—a figure substantially higher than any of the rates obtained for unparasitized blood, normal or anemic.

To make such values really comparable with those obtained for unparasitized blood it is necessary to know both the parasite count, and the proportions present of parasites of different sizes. Silverman and his colleagues[7] distinguished 3 types: Merozoites freshly formed by segmentation they called "Type A." Somewhat larger stages, having up to 4 masses of chromatin, they designated as "Type B," and all others were called "Type C." They found the surface areas of these 3 types to be approximately 1, 12, and 48 square micra.

Using this scheme of classification, we determined their hourly glucose consumption rates to be about 3.00×10^{-11} mg, 3.60×10^{-10} mg, and 1.44×10^{-9} mg respectively. An "average" parasite (one having the mean of these 3 areas) consumed glucose about 14 times as fast as an adult erythrocyte and twice as fast as a reticulocyte. The data on which these and other calculations were based is summarized in Table I.

From these data the glucose consumption rates per square micron of surface area may also be calculated, both for erythrocytes and parasites. We found this to be 3.42×10^{-13}

‖ This involved the use of the formula

$$\text{mg glucose per hour per ml} = E(AX \times RX')$$

in which

$$A = \frac{\text{adult erythrocytes}}{\text{total erythrocytes}}$$

$$E = \frac{\text{erythrocytes per mm}^3}{10^6}$$

$$R = \frac{\text{reticulocytes}}{\text{total erythrocytes}}$$

X = hourly glucose consumption (mg) of 10^9 red cells

X' = hourly glucose consumption (mg) of 10^9 reticulocytes

[7] Silverman, M., Ceithaml, J., Taliaferro, L. G., and Evans, E. A., *J. Inf. Dis.*, 1944, 75, 212.

TABLE I.
Influence of Riboflavin on the Growth of Tubercle Bacillus.

Medium, character of	Control, no riboflavin	Riboflavin, % conc. 0.25	0.5	1.0
	% transmission with air blank as reference			
Vit. free	68.5	66.4	67.1	66.3
+ Vit. A	61.8	61.1	—	—
+ Vit. A + Biotin	58.4	61.4	—	—
+ Vit. A + Thiamine	58.8	58.6	—	—
+ Vit. A + Biotin + Thiamine	59.3	57.2	—	—

sence of riboflavin (Boissevain *et al.*[4]; Street and Reeves[5]) they may remove riboflavin from the host during the multiplication and thus bring about a deficiency condition. The present experiment was carried out to determine first whether the addition of riboflavin to the medium would promote the growth of tubercle bacilli and second whether there would be a loss of riboflavin from the medium during the growth of the organism.

Procedure. Human tubercle bacilli of a slightly virulent strain TBI† were used. The culture medium was made according to the formula of Dubos and Middlebrook[6] with the following modifications: Twenty ml of hydrolyzed vitamin free casein solution (1 ml corresponds to 100 mg casein) were used instead of 2 g enzymatic digest of casein. Tween 80 was purified according to the method of Davis[7] while bovine albumin was omitted.

For the study of the effect of riboflavin on the growth of tubercle bacillus 10 ml portions of the medium were distributed in 50 cc Erlenmeyer flasks. For each series 4 to 6 flasks were used as controls while a similar number was used for each of 3 different levels of riboflavin, namely, 0.025 mg, 0.05 mg, and 0.1 mg per 10 ml. After the addition of riboflavin or other vitamin solutions, the final volume of liquid in each flask was made up to the same amount by the addition of sterile distilled water. To prevent the destruction of riboflavin by light the flasks containing the riboflavin solution were covered with black cloth or paper. All flasks containing the medium were incubated for 24 hours to see if any was contaminated. One-tenth ml of a liquid tubercle bacillus culture 7 to 10 days old was added to each flask. The amount of bacterial inoculum was estimated to be 0.0014 mg, 0.014 mg and 0.14 mg for different series of experiments. After 4 to 7 days incubation at 37°C the degree of growth was determined turbidimetrically by means of a Coleman Junior Spectrophotometer. The metal screw-capped test tubes used for the reading were read first with air as a reference. The wave length was set at 560 mμ.

For the study on the loss of riboflavin from the medium, 4.0 mg of riboflavin in 80 ml of 0.02 N acetic acid solution were added to 1 liter of medium so that each ml of the medium contained about 4 γ of riboflavin. One hundred ml were distributed in each of 10 flasks, which were protected from light by wrapping with black cloth or paper. After incubation for 24 hours, to determine if the medium rethat the glucose consumed by parasitized blood (W_t) is the sum of that utilized by adult red cells (W_A), reticulocytes (W_R), and parasites (W_P) in unit time.

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[4] Boissevain, C. H., Drea, W. F., and Schultz, H. W., PROC. SOC. EXP. BIOL. AND MED., 1938, 39, 481.

[5] Street, H. R., and Reeves, R. F., PROC. SOC. EXP. BIOL. AND MED., 1940, 44, 641.

† Originally came from the Bureau of Animal Industry.

[6] Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberculosis*, 1947, 54, 334.

[7] Davis, B. D., *Arch. Biochem.*, 1947, 15, 359.

W_A = number of red cells in 1 ml blood × hourly glucose consumption of 1 red cell or $(10^9 \times A \times E) \times (4.34 \times 10^{-11}) = 4.34AE \times 10^{-2}$ mg

W_R = number of reticulocytes in 1 ml blood × hourly glucose consumption of 1 reticulocyte or $(10^9 \times R \times E) \times (3.06 \times 10^{-10}) = 3.06RE \times 10^{-1}$ mg

W_P = total square micra of parasite surface area × hourly glucose consumption per square micron or $(PE \times 10^5 (a + 12b + 48c)) \times (3.00 \times 10^{-11}) = 3.00PE \times 10^{-6} (a + 12b + 48c)$ mg

Therefore

$$W_t = (4.34AE \times 10^{-2}) + (3.06RE \times 10^{-1}) + (3PE \times 10^{-6}) (a + 12b + 48c) = 4.34E \times 10^{-2} (A + 7.05R + 6.92P \times 10^{-5} (a + 12b + 48c))$$

Discussion. In making the calculations and constructing the formula above it has been assumed that (1) the blood cells are in an essentially normal environment for at least the first 30 minutes, and therefore metabolize normally, (2) the metabolism of leucocytes and thrombocytes is normal and approximately constant in each sample, (3) the levels of any hormones influencing red cell metabolism are constant, or negligible in effect, (4) the metabolism of the infected red cell is undisturbed by the presence of the parasite, and (5) the metabolism of the gametocytes is the same, or not greatly different from that of asexual forms of like size. The fourth and fifth assumptions may be open to question, but it has so far not been possible to put them to experimental test.

Despite certain sources of error inherent in the experimental procedures, such as the counting technics, agreement between the experimentally determined and the values predicted by the formula has been close, the mean deviation between the two having been only ± .036 mg glucose per ml per hour.

Summary and Conclusions. A study of the glucose requirements of *Plasmodium gallinaceum* has been made, and formulae derived for predicting the hourly glucose consumption rates of parasitized and unparasitized blood. These should be useful in predicting the amounts of glucose required for cultures and indicative of the *in vivo* drain on carbohydrate stores. Normal chicken blood was found to have an hourly glucose consumption rate of approximately 8.7 mg per 100 ml, and values as high as 74.7 mg per 100 ml were observed for parasitized blood. The glucose consumption per square micron per hour was 3.42×10^{-13} mg, 2.41×10^{-12} mg, and 3.00×10^{-11} mg for adult red cells, reticulocytes, and parasites respectively.

17001

Riboflavin and Growth of Tubercle Bacilli.

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From the Departments of Agricultural Bacteriology and Biochemistry, University of Wisconsin, College of Agriculture, Madison, Wis.

It was reported previously (Hou;[1] Farber and Miller[2]) that riboflavin deficiency was common among patients suffering from tuberculosis and that phlyctenular conjunctivitis or keratitis frequently observed in tuberculous patients responded to riboflavin therapy (Hou[3]). It was postulated (Hou[1]) that although tubercle bacilli will grow in the ab-ation it was possible to obtain purified s showing similar characteristics as reported by Foster. It appears there- nat during the growth of tubercle bacil- boflavin may be converted into lumi- e. This would furnish an explanation, st partly, for the high incidence of ribo- deficiency among tuberculous patients. finding that addition or increased con- tion of riboflavin in the medium did not nce the growth of tubercle bacillus lead us to believe that there is no r of promoting the growth of the organ- by therapeutic administration of ribo-

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* On leave from The Institute of Nutrition, China, and on a traveling fellowship of the World Health Organization of the United Nations.

[1] Hou, H. C., *Chinese J. Med.*, 1943, **62**, 181.

[2] Farber, J. E., and Miller, D. K., *Am. Rev. Tuberc.*, 1943, **48**, 412.

[3] Hou, H. C., *Chinese Med. J.*, 1940, **58**, 616.

nmary. A slightly virulent strain of n tubercle bacillus was cultured in a in free medium or in media to which avin with or without other vitamins and biotin were added.

It was found that the presence or absence of riboflavin made no difference in the growth of the tubercle bacillus. On the other hand during the growth of the organism there was found a slow but steady disappearance of riboflavin from the medium. The presence of lumichrome crystals in the media containing riboflavin after growth of the tubercle bacillus indicated that the loss of the vitamin was due to conversion to lumichrome.

The writer is indebted to Dr. D. W. Watson and Mr. R. J. Heckly of the Department of Agricultural Bacteriology for criticism and assistance in the execution of the bacteriological technics, to Dr. C. A. Elvehjem, Department of Biochemistry, for his advice and encouragement in this work, and to Messrs. M. Moinuddin and C. C. Clayton for assistance in the riboflavin assays.

17002

Recovery of Psittacosis Virus from Chicks Hatched from Inoculated Eggs.

DORLAND J. DAVIS AND JOHN E. VOGEL.

From the Laboratory of Infectious Diseases, Microbiological Institute, National Institutes of Health, Bethesda, Md.

vestigations[1] of psittacosis infections in acine and other birds have shown that e infection with the virus (*Miyagawanel- ittaci*) is encountered in young birds, even lings. This has suggested that infection ansmitted from latently infected adults er directly to the nestling or congenitally ugh the egg.[1] Furthermore the virus has n reported to have been recovered from es and egg yolk in the oviduct of para- s.[1] The possibility exists therefore, that genital transmission may be important in emination of the virus among psittacine ls, pigeons, and other susceptible species. In this laboratory an effort was made to ure experimental evidence concerning trans- ssion of *M. psittaci* by the egg in avian ecies. Isolations of virus were attempted om chicks hatched from eggs which had been inoculated during various stages of embryonic development.

Although psittacosis infection in chickens is not known to be widespread this species has been found naturally infected[2] and the ready accessibility of chicken eggs and familiarity with technics for handling the infection in them made this species a logical choice for preliminary work.

The strain of virus used, No. 4, had been isolated in this laboratory from a psittacine bird (*Aratinga* sp.) and maintained by serial egg passage for at least 14 transfers. It was approximately 10^4 times more lethal for white mice by intracranial inoculation than by the intraperitoneal route and was well adapted to growth in allantoic cavity and yolk sac of the developing chick embryo.

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[1] Meyer, K. F., *Medicine*, 1942, **21**, 175.

[2] Meyer, K. F., and Eddie, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 522.

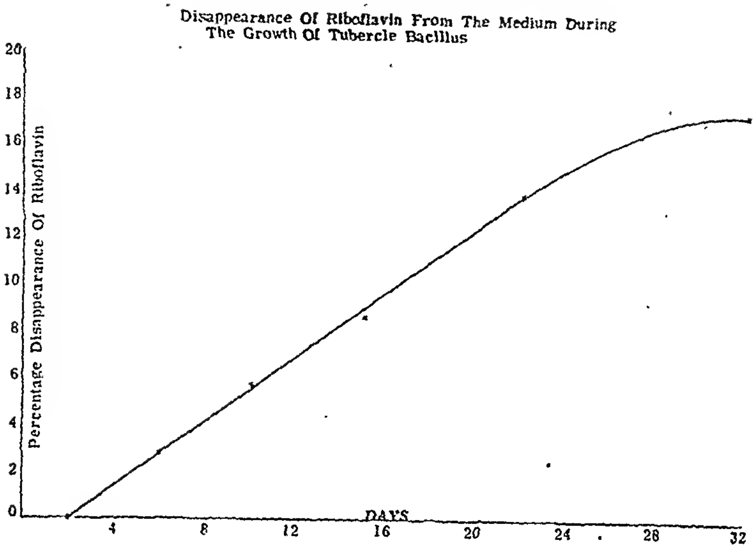

FIG. 1.

mained sterile, one-half the number of flasks were kept as controls while the remaining were inoculated with tubercle bacillus. All flasks were incubated at 37°C. At intervals of 3 days to one week 5 ml samples were removed from each flask and autoclaved at 15 lb for 10 minutes. Smears were made at each sampling to exclude any contaminated organism. If present the sample was discarded. The samples were subsequently centrifuged at high speed for 10 minutes to remove all solid particles and/or bacteria. The supernatant fluid was used for the analysis of riboflavin content, which was determined microbiologically according to a modified method of Snell and Strong[8] and fluorometrically by a composite procedure of several methods.[8]

Results and Discussion. Effect of riboflavin on growth of tubercle bacilli. The results of the study are summarized in Table I. Each figure represents the average of 10 to 20 different sets of readings. With the medium free from riboflavin the growth of tubercle bacilli was similar to that in media containing different levels of riboflavin, namely, 0.25, 0.5 and 1.0%. The addition of vitamin A, thiamine and biotin, individually or together, to the medium with or without riboflavin also did not influence the growth of the organism.

Effect of growth of tubercle bacillus on the level of riboflavin in the medium. The results of this study are shown in Fig. 1. During the first 22 days of incubation the percentage of disappearance of riboflavin from the medium plotted against time in days gave a straight line. After that time the curve began to flatten off. The disappearance of riboflavin apparently was proportional to the growth of the organisms. By the fourth week the growth had reached its maximum and the disappearance of riboflavin became correspondingly slower. On the whole the loss of riboflavin, however, was not as great as one would expect with the abundance of growth. It is conceivable that some dead organisms might have contributed some riboflavin to the medium. Foster[9] recently reported that riboflavin could be converted into lumichrome by an organism which he named *Pseudomonas riboflavinus nov. sp.* The lumichrome crystals were needle-like in appearance and could be isolated and identified by a characteristic absorption spectrum. In the present experiment similar crystals were first observed in stained smears and then in unstained liquid smears. Using Foster's technic of isolation it was possible to obtain purified crystals showing similar characteristics as those reported by Foster. It appears therefore that during the growth of tubercle bacillus riboflavin may be converted into lumichrome. This would furnish an explanation, at least partly, for the high incidence of riboflavin deficiency among tuberculous patients.

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[8] Association of Vitamin Chemists, Inc., Interscience Publishers, Inc., New York, 1947.

[9] Foster, J. W., *J. Bact.*, 1944, 47, 27.

The finding that addition or increased concentration of riboflavin in the medium did not influence the growth of tubercle bacillus would lead us to believe that there is no danger of promoting the growth of the organism by therapeutic administration of riboflavin.

Summary. A slightly virulent strain of human tubercle bacillus was cultured in a vitamin free medium or in media to which riboflavin with or without other vitamins and biotin were added.

It was found that the presence or absence of riboflavin made no difference in the growth of the tubercle bacillus. On the other hand during the growth of the organism there was found a slow but steady disappearance of riboflavin from the medium. The presence of lumichrome crystals in the media containing riboflavin after growth of the tubercle bacillus indicated that the loss of the vitamin was due to conversion to lumichrome.

The writer is indebted to Dr. D. W. Watson and Mr. R. J. Heckly of the Department of Agricultural Bacteriology for criticism and assistance in the execution of the bacteriological technics, to Dr. C. A. Elvehjem, Department of Biochemistry, for his advice and encouragement in this work, and to Messrs. M. Moinuddin and C. C. Clayton for assistance in the riboflavin assays.

17002

Recovery of Psittacosis Virus from Chicks Hatched from Inoculated Eggs.

DORLAND J. DAVIS AND JOHN E. VOGEL.

From the Laboratory of Infectious Diseases, Microbiological Institute, National Institutes of Health, Bethesda, Md.

Investigations[1] of psittacosis infections in psittacine and other birds have shown that active infection with the virus (*Miyagawanella psittaci*) is encountered in young birds, even nestlings. This has suggested that infection is transmitted from latently infected adults either directly to the nestling or congenitally through the egg.[1] Furthermore the virus has been reported to have been recovered from ovaries and egg yolk in the oviduct of parakeets.[1] The possibility exists therefore, that congenital transmission may be important in dissemination of the virus among psittacine birds, pigeons, and other susceptible species.

In this laboratory an effort was made to secure experimental evidence concerning transmission of *M. psittaci* by the egg in avian species. Isolations of virus were attempted from chicks hatched from eggs which had been inoculated during various stages of embryonic development.

Although psittacosis infection in chickens is not known to be widespread this species has been found naturally infected[2] and the ready accessibility of chicken eggs and familiarity with technics for handling the infection in them made this species a logical choice for preliminary work.

The strain of virus used, No. 4, had been isolated in this laboratory from a psittacine bird (*Aratinga* sp.) and maintained by serial egg passage for at least 14 transfers. It was approximately 10^4 times more lethal for white mice by intracranial inoculation than by the intraperitoneal route and was well adapted to growth in allantoic cavity and yolk sac of the developing chick embryo.

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[1] Meyer, K. F., *Medicine*, 1942, **21**, 175.

[2] Meyer, K. F., and Eddie, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 522.

TABLE I.
Recovery of *Miyagawanella psittaci* from Chicks Hatched from Eggs Inoculated after Various Periods of Incubation.

Days, incubation	No. eggs inoculated in yolk sac	No. eggs inoculated in allantoic cavity	No. chicks hatched	Recovery of virus from chicks by age in days*					
				0-1	2-9	10-19	20-29	30-39	Total
0	83		14	0/1	0/5				0/6
4	56		12	0/4	0/8				0/12
7	53	32	12	0/4	0/2				0/6
8	126	48	8	1/8†					1/8
9	67		12	0/4	0/5	0/2			0/11
10	65		35	0/10	0/9	0/7	0/8		0/34
11	31		16	0/4	0/5	0/3			0/12
12	57		14	0/5	0/2	0/2			0/9
14	202		88	14/20	8/20	2/13	1/15	0/10	25/78
15	109		82	4/15	1/15	2/17	0/10	0/13	7/70
16	58		39	0/2	3/7	1/10	0/18		4/37
18		31	26	1/5	0/5	0/3	0/13		1/26
Total	907	111	358	20/85	12/83	5/57	1/64	0/23	38/304 (12.5%)

* No. chicks infected/No. chicks examined.
† Infected chick hatched from egg inoculated in allantoic cavity.

The eggs were held at 38°C throughout the entire time of incubation. After hatching the chicks were kept in a brooder for at least one week.

After being incubated for various periods from 0 to 18 days the developing embryos were inoculated into the yolk sac or allantoic cavity with .25 ml of a dilution of virus in physiological saline found by previous titration to kill approximately one half the embryos. Nearly all chicks which hatched were autopsied and virus isolations attempted at various time intervals from 1 to 39 days after hatching. The spleen, liver, and kidney of each chick were ground together in a mortar, suspended in physiological saline and injected intracranially into 5 mice. In some instances these organs were suspended and inoculated separately. The microscopic demonstration of typical clusters of elementary bodies in impression smears taken from the brains of mice dying from 3 to 7 days after inoculation and stained by the Machiavello method was taken as evidence of the presence of *M. psittaci* in the chicks.

The table presents data concerning 1018 eggs inoculated in the yolk sac or allantoic cavity after various periods of incubation. Three hundred fifty-eight chicks were hatched. Of these 304 were examined and the presence of *M. psittaci* was demonstrated in 38 of them (12.5%). The table also shows the number of chicks infected with virus in relation to the number examined for various ages in days. A few more than half of the isolations were from chicks autopsied within 24 hours after hatching; some of these chicks were moribund. The proportion of infected chicks to those examined decreased with the advancing age of the chick. The oldest chick from which virus was isolated was 22 days old, and the egg from which it had hatched had been inoculated on the 14th day of incubation.

With the exception of the moribund recently hatched chicks, all appeared in good condition and the only abnormality noted at autopsy was an enlarged spleen in a few instances which was not correlated with the recovery of virus. Virus was recovered from separate suspensions of the kidney, spleen, or liver of 7 and 14 day old chicks. Sixteen embryos dying from the 18th to 21st day of development which had been inoculated on the 8th day were examined and virus was recovered from the internal organs of 9 of them.

Summary. The data here presented indicates that chicks will hatch from eggs which have been infected with *M. psittaci* during the course of embryonic development and will survive in apparently good condition while harboring the virus in the organs for at least

22 days after hatching. While these experiments do not furnish evidence for the congenital transmission of the virus in the chicken, it is possible that in a more susceptible species the virus could be carried more effectively through the developmental and hatching period after either experimental or congenital infection.

17003 P

Studies of the "Thrombin" Effect of Fresh Serum.*

RALPH F. JACOX AND ROBERT P. BAYS. (Introduced by W. S. McCann.)

From the University of Rochester School of Medicine and Dentistry, and the Clinic of the Strong Memorial and Rochester Municipal Hospitals, Rochester, N. Y.

A factor capable of producing prothrombin conversion has been described in a previous report.[1] This factor can be easily demonstrated after thromboplastin is added to serum which contains no thrombin and only traces of prothrombin. The resultant mixture of serum and thromboplastin causes rapid coagulation of a 0.01 M oxalated plasma.[†] Suitable control studies revealed that this factor (designated "prothrombin-converting factor"), is not thrombin, but a substance which requires prothrombin to mediate a coagulation effect on fibrinogen. Furthermore, the coagulation of 0.01 M oxalated plasma by the serum-thromboplastin mixture could not be explained as a separate action of either component.

A study has been made of the coagulation effect of *fresh serum alone* on 0.01 M oxalated plasma. It has been assumed by other workers,[2,3] that the residual clotting action of fresh serum is related to thrombin. After conversion of fibrinogen to fibrin, thrombin was believed to combine with albumin to form inactive metathrombin,[2] or that thrombin was destroyed by an enzyme.[3] Our results reveal that the residual coagulating action of fresh serum is almost entirely due to "prothrombin-converting factor". This data confirms and extends the observations of Bordet and Gengou[4] who showed that serum coagulated whole oxalated plasma much more rapidly than it did oxalated plasma from which prothrombin was removed by adsorption with tricalcium phosphate.

The coagulation effect of fresh serum was studied by the following methods. Blood was withdrawn from human donors and placed in glass tubes with internal dimensions of 1.0 x 10.0 cm. Ordinary care was used in collecting the specimens to avoid contamination with tissue thromboplastic substances. Within 15 minutes of collection, the tubes containing the blood were placed in a 26°C water bath. Approximately 50 minutes later the serum was separated from the clot. One-tenth cubic centimeter of serum (kept at 26°C) was pipetted into 0.1 cc of a 0.01 M oxalated plasma and the coagulation time determined.

It was observed that serum, which coagulated plasma rapidly when tested within 70 minutes of the time of collection from the donor, was incapable of coagulating a buffered[‡] fibrinogen solution (200-300 mg %

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* Research was carried out under a grant for the study of Rheumatic Fever made by the Masonic Foundation for Medical Research and Human Welfare.

[1] Jacox, Ralph F., to be published in *J. Clin. Invest.*

[†] All plasma used in these experiments was prepared by adding 9.0 cc of whole blood to 1.0 cc of 0.1 M potassium oxalate solution.

[2] Quick, A. J., *Am. J. Physiol.*, 1938, **123**, 712.

[3] Glazko, A. J., and Ferguson, J. H., *J. Gen. Physiol.*, 1940, **24**, 169.

[4] Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1904, **18**, 98.

[‡] Collidine buffer (pH 7.3) was used in all experiments.[5]

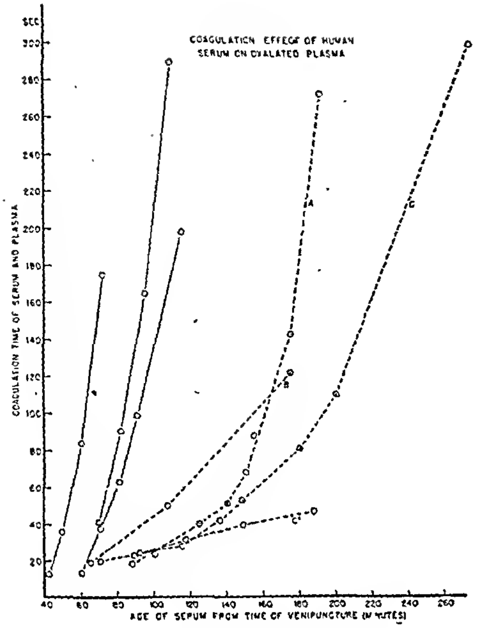

FIG. 1.

Human serum was added to 0.01 M oxalated plasma at designated intervals (abscissa). The coagulation time of serum and plasma is recorded on the ordinate. Normal serum is depicted by unbroken lines; serum from patients receiving dicumarol by broken lines. C¹ serum (obtained from a patient receiving dicumarol with prothrombin less than 20 per cent of normal) had the most prolonged coagulation effect for oxalated plasma.

concentration). The fibrinogen solution§ and the plasma were equally reactive to thrombin since each coagulated with the same speed when bovine thrombin (Upjohn Co.) was added. Fig. 1 demonstrates the coagulating effect of serum when it is added to oxalated plasma. Immediately after separation of serum from the clot, coagulation of oxalated plasma was prompt (12-40 sec.). The coagulation effect of serum from normal individuals rapidly disappeared until no clotting activity remained 100-120 minutes after the time of venipuncture. Serum obtained from patients receiving dicumarol (Fig. 1—broken line) had a significantly decreased rate of degradation of the coagulating substance. As the plasma prothrombin decreased, the "prothrombin-converting factor" rate of degradation was proportionately decreased. These results are in accord with a previous observation[1] that thromboplastin activation of serum from patients receiving dicumarol, produced a slowly decaying action of the freed "prothrombin-converting factor".

Since the degradation rate of "prothrombin-converting factor" in sera of dicumarolized subjects proceeds slowly, it has been possible to obtain sera which had little loss of coagulation effect for 0.01 M oxalated plasma, 30-60 minutes from the time of separation from clotted whole blood. Such a serum was prepared by withdrawing blood from rabbits who had been given large amounts of dicumarol 24-28 hours before collection of the serum. This serum was utilized to produce the results shown in Fig. 2. The active, slowly degrading rabbit serum was added to 0.01 M oxalated

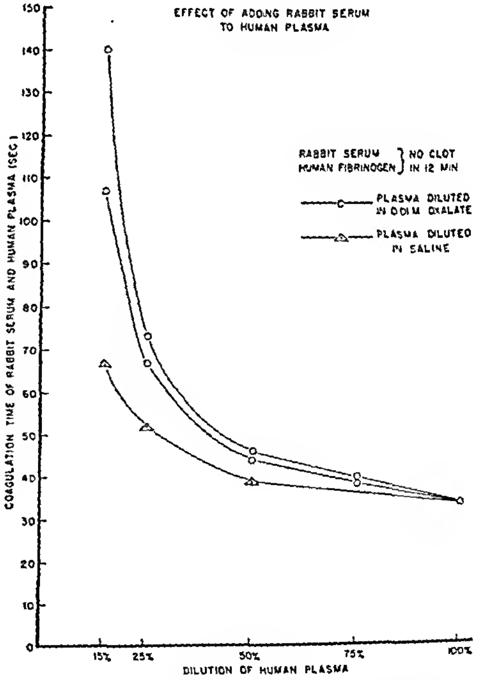

FIG. 2.

Serum obtained from a dicumarolized rabbit was added to varying dilutions of two normal human plasmas (upper 2 curves). The coagulating effect of rabbit serum is dependent upon prothrombin (fibrinogen did not clot in 12 minutes). The lower curve represents dilution of one of the two plasmas in saline rather than 0.01 M potassium oxalate, following which rabbit serum was added as described.

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[5] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 33.

§ Fibrinogen was supplied through the generosity of Armour and Company.

human plasma and to dilutions of plasma made in 0.01 M oxalated 0.9% sodium chloride solution. The coagulation time for each dilution of plasma was then determined after addition of active rabbit serum. It will be observed (Fig. 2) that the activity of the "prothrombin-converting factor" in rabbit serum is dependent upon prothrombin concentration of the plasma. A curve can therefore be constructed (Fig. 2) which resembles the reaction curve obtained in the one stage prothrobin test of Quick.[6] A similar curve can be obtained by substitution of rabbit for human plasma. This suggests that rabbit and human plasma contain nearly identical amounts of prothrombin.

It will be observed furthermore (Fig. 2), that plasma diluted in 0.9% sodium chloride solution rather than 0.01 M oxalated sodium chloride solution, produced faster coagulation in the diluted fractions when the active serum was added. This suggests that the "prothrombin-converting factor" may be partially inhibited by the oxalate ion (calcium effect?) or that "prothrombin-converting factor" may be auto-catalytically activated from plasma.

Discussion. The residual coagulating power of serum, after complete coagulation of whole blood has taken place, is not related to thrombin but rather to a "prothrombin converting factor". It is assumed that this factor is initially activated through the action of plasma thromboplastin and platelets. The "prothrombin-converting factor" then causes thrombin production by reacting with prothrombin. The thrombin quickly disappears after fibrin is formed, whereas the "prothrombin-converting factor" can be easily measured in the serum for at least 100 minutes from the time of venipuncture. In sera of patients who receive dicumarol, the "prothrombin-converting factor" effect is greatly prolonged over that observed in normal serum.

An accurate analysis of plasma prothrombin concentration can be made by use of a relatively stable "prothrombin-converting factor" obtained from rabbits receiving dicumarol. This technic of assay reproduced results obtained with the one stage determination of prothrombin by Quick's method.[6]

Owren,[7] who described factor VI (which we believe to be the same as "prothrombin-converting factor"), has reasonably credited Bordet and Gengou[4] with the first demonstration of this forgotten concept in blood coagulation.

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[6] Quick, A. J., *The Hemorrhagic Diseases and Physiology of Hemostasis* (Thomas, 1942).

[7] Owren, P., *Acta Med. Scand.*, 1947, Supp. 194.

17004

Evaluation of Dubos' Solid Medium Containing Penicillin in the Isolation of Tubercle Bacilli.

J. W. Smith, J. Humiston, W. P. Creger, and W. M. M. Kirby.
(Introduced by A. L. Bloomfield.)

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Following the observation by Dubos and Davis that a liquid medium containing Tween 80 would allow rapid, submerged growth of mammalian tubercle bacilli,[1] the diagnostic potentialities of this medium have been explored in many laboratories.[2,3] The results have in general been favorable, but two disadvantages have become apparent. First, no satisfactory agent has been found to restrain contaminating organisms which are not destroyed by treatment with alkali. Penicillin is not suitable for this purpose, since in the presence of Tween it causes inhibition of tubercle bacilli in concentrations greater than 1 or 2 units per cc.[4] The other disadvantage is the necessity of staining the organisms growing in the medium to be certain they are acid-fast bacilli. If only a few tubercle bacilli are present, and particularly when there are contaminants, it is often tedious and difficult to demonstrate their presence.

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[1] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[2] Foley, G. E., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 298.

[3] Foley, G. E., *J. Lab. and Clin. Med.*, 1947, **32**, 842.

More recently Dubos and his associates have described a solid medium containing oleic acid, albumin, and agar, which is relatively clear and transparent, and supports the growth of small numbers of tubercle bacilli.[5] In contrast to the liquid Tween medium, penicillin can be added in concentrations of 100 units per cc without inhibiting the growth of tubercle bacilli.[4] Virulent tubercle bacilli grow on this medium in a characteristic cord-like manner, easily observed under the low power of the microscope, which distinguishes them from avirulent tubercle and from the colonial forms of other bacteria.[6]

Because of these apparent advantages, a study was undertaken to compare the solid oleic acid-albumin medium with a standard egg-potato medium in the isolation of tubercle bacilli from clinical specimens, and to determine the effectiveness of penicillin in restraining contaminants.

Methods and Materials. Each specimen was digested with an equal volume of 4% NaOH for one hour at 37°C, with a 10 minute period of agitation in a shaking machine. Smears were made of the neutralized, centrifuged sediment, which was suspended in 0.5 cc of saline, and 2 loopfuls of the suspensions were inoculated on each medium.

The media employed were: (1) solid oleic acid-albumin, (2) solid oleic acid-albumin containing 100 units per cc of penicillin, (3) a widely used egg-potato medium containing 0.02% malachite green.[7] The media were allowed to solidify in one ounce prescription bottles, or small (1.5 by 5 cm) Petri dishes sealed with scotch tape. Only one sample of each medium was inoculated from any one specimen.

Results. Tubercle bacilli grew on one or more of the media in 156 of 300 specimens cultured. Of the 156 positive specimens, 61 were sputa, 91 urines, 2 gastric washings, and 2 were pus from abscesses. Comparative results are presented in Table I. The most striking observation, evident from the second column, is that positive cultures were obtained 10 days sooner, on the average, with oleic acid-albumin medium than with the egg-potato. There was no evidence that growth was delayed by the addition of penicillin to the Dubos medium.

TABLE I.
General Comparison of Positive Cultures Obtained on Three Media.

	No. of cultures positive	Avg. No. days required to obtain positive culture
Total No. of specimens positive by all methods	156	
Oleic acid-albumin medium	91	14
Oleic acid-albumin medium plus penicillin	134	14.6
Egg-potato medium	120	24.4

TABLE II.
Analysis of Results from the Standpoint of Positive and Negative Cultures Obtained with Each Medium.

	No. of cultures
Positive oleic acid plus penicillin Negative plain oleic acid	55
Positive plain oleic acid Negative oleic acid plus penicillin	8
Positive oleic acid plus penicillin Negative egg-potato	20
Positive egg-potato Negative oleic acid plus penicillin	14

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[4] Kirby, W. M. M., and Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1947, 66, 120.

[5] Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, 56, 334.

[6] Middlebrook, G., Dubos, R. J., and Pierce, C., *J. Exp. Med.*, 1947, 86, 175.

[7] American Trudeau Society, *Am. Rev. Tuberc.* (Abstracts), 1946, 54, Nos. 4-5.

From the standpoint of total positive cultures, the solid oleic acid-albumin medium containing penicillin appeared to be slightly superior to the egg-potato, there being 134 positives with the former, and 120 with the latter. This slight superiority is also apparent in Table II, where it can be seen that the Dubos medium containing penicillin was positive in 20 instances when the egg-potato was negative, whereas the reverse situation, (positive egg-potato, negative Dubos plus penicillin) occurred with 14 specimens.

The Dubos medium was much more efficient with the addition of penicillin than without. In 32 instances in which recovery of tubercle bacilli was prevented by the overgrowth of contaminants on the plain Dubos medium, the contaminants were suppressed on the penicillin-containing medium, and positive cultures were obtained. In one of the 8 instances in which the plain Dubos plate was positive, while that containing penicillin was negative, the tubercle bacillus was found to be inhibited by a concentration of only 10 units per cc of penicillin.

Comment. The present study indicates that Dubos' solid oleic acid-albumin medium, containing 100 units per cc of penicillin, is slightly more sensitive than a standard egg-potato medium in detecting tubercle bacilli in specimens obtained from clinical sources, and has the advantage of giving positive results 10 days earlier, on the average.

Another desirable feature is that the cord-like growth of virulent tubercle bacilli is highly characteristic on the transparent oleic acid-albumin plates; the colonies can be recognized at a glance by focusing on the agar surface with the low power of the microscope. This ability to differentiate virulent from avirulent tubercle bacilli on simple morphological grounds will probably obviate the necessity of inoculating guinea pigs; to our knowledge, no avirulent strains have been discovered which produce typical cord-like colonies.

Penicillin appears to be a highly satisfactory agent for inhibiting the growth of contaminants on the oleic acid-albumin medium. It should be noted that tubercle bacilli isolated from the urine in one patient were inhibited by 10 units per cc of penicillin; in the 134 other instances growth of the tubercle bacilli was in no way affected by a concentration of 100 units per cc of penicillin.

The widespread notion that Dubos media provide isolation from clinical specimens in 10 days, as opposed to 4 to 8 weeks with egg-potato media, is incorrect. Actually, cultures usually become positive on Dubos media from 7 to 14 days earlier than on egg-potato, regardless of the length of time required for growth to appear. With small inocula, several weeks are required; with larger inocula, growth is often visible in 7 to 14 days.

Summary and Conclusions. Of 300 clinical specimens, 134 were positive for tubercle bacilli on Dubos' solid oleic acid-albumin medium containing 100 units per cc of penicillin, and 120 were positive on a standard egg-potato medium. Without the presence of penicillin to restrain contaminants, only 91 cultures were positive on the Dubos medium. The Dubos medium was also considered superior because of the ease with which growth could be observed on the transparent agar, and because of the characteristic cord-like appearance of virulent tubercle bacilli, which apparently distinguishes them from avirulent forms, and from other organisms.

17005

Influence of Intestinal Bacteria on Synthesis of Nicotinic Acid from Tryptophan.

JAMES M. HUNDLEY. (Introduced by J. G. Wooley.)

From the Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Md.

Several investigators have presented suggestive but not conclusive evidence that intestinal bacteria play an important role in synthesizing nicotinic acid, making this vitamin available to the host. This subject has been reviewed elsewhere.[1]

Since it has been shown that tryptophan can substitute for nicotinic acid in the diet of most species,[2-7] and since tryptophan acts by increasing the synthesis of nicotinic acid,[8-16] it has been a natural assumption that the synthesis of nicotinic acid from tryptophan may involve the intestinal bacteria.

Ellinger and Abdel Kader[14] have reported that succinylsulfathiazole greatly reduced the urinary output of N'-methylnicotinamide when tryptophan was administered. From this and other evidence they concluded that the intestinal bacteria were involved in the conversion of tryptophan to nicotinic acid. However, Spector[12] in a somewhat similar type of experiment was led to the opposite conclusion. Junqueira and Schweigert[17] found that succinylsulfathiazole would not interfere with the conversion of tryptophan to nicotinic acid under certain conditions but would under others.

Dann and Handler[18] and Levy and Young[19] have shown that the bacteriologically sterile chick embryo can synthesize nicotinic acid. Schweigert *et al.*[20] have shown that tryptophan will increase the nicotinic acid content of the chick embryo. These experiments demonstrate that at least under certain conditions, nicotinic acid can be synthesized by the tissues independent of bacterial action.

It is the purpose of this communication to present evidence demonstrating that synthesis of nicotinic acid from tryptophan is not dependent on the intestinal bacteria in the rat.

Experimental methods. Male rats weighing approximately 250 g were maintained on a purified "nicotinic acid free" diet consisting of 12% casein, 81% sucrose, 3% corn oil, 4% minerals and the usual vitamins except nicotinic acid as described elsewhere.[10]

After at least one week on this diet, 24 hour urine specimens were collected from each rat and the basal output of N'-methlynicotinamide determined. Each rat was then given subcutaneously 100 mg 1 (–) tryptophan dissolved in 10 cc of 0.85% sodium chloride solution and the urinary N′-methylnicotinamide output in the following 24 hours determined. All solutions were sterilized by boiling.

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[1] Ellinger, J., *J. Am. Med. Assn.*, 1946, **130**, 668.

[2] Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

[3] Briggs, G. M., *J. Biol. Chem.*, 1945, **161**, 749.

[4] Wooley, J. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 315.

[5] Singal, S. A., Sydenstricker, V. P., and Littlejohn, Julia M., *J. Biol. Chem.*, 1948, **176**, 1051.

[6] Schweigert, B. S., Pearson, P. B., and Wilkening, M. C., *Arch. Biochem.*, 1947, **12**, 139.

[7] Lueke, R. W., McMillen, W. N., Thorp, F., Jr., and Tull, C., *J. Nutr.*, 1947, **33**, 251.

[8] Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573; *ibid.*, 1947, **171**, 203.

[9] Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1946, **163**, 343.

[10] Hundley, J. M., *J. Nutr.*, 1947, **34**, 253.

[11] Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

[12] Spector, H., *J. Biol. Chem.*, 1948, **173**, 659.

[13] Sarrett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, 1947, **167**, 293.

[14] Ellinger, P., and Abdel Kader, M. M., *Nature*, 1947, **160**, 675.

[15] Perlzweig, W. A., Rosen, F., Levitas, N., and Robinson, J., *J. Biol. Chem.*, 1947, **167**, 511.

[16] Heidelberger, C., Abraham, E. P., and Lepkovsky, S., *J. Biol. Chem.*, 1948, **176**, 1461.

[17] Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, 1948, **175**, 535.

[18] Dann, W. J., and Handler, P., *J. Biol. Chem.*, 1941, **140**, 935.

[19] Levy, M., and Young, N. F., *J. Biol. Chem.*, 1948, **176**, 185.

[20] Schweigert, B. S., German, H. L., and Garber, M. J., *J. Biol. Chem.*, 1948, **174**, 383.

The animals were then matched in control and experimental groups according to their weight and to the amount of N′-methylnicotinamide excreted in response to tryptophan. The control animals were subjected to the same surgical and other procedures as the experimental animals, except that they received only saline subcutaneously while the experimental animals received the same amount of saline containing the tryptophan. A rest period of at least one week was allowed between the test dose of tryptophan and the operative procedures.

In some of the animals the entire intestine, excluding the stomach, was removed. In the remainder, only the stomach was excised. Clean but not aseptic technic was observed. The bowel stumps were simply ligated. No effort was made to anastomose esophagus and duodenum in the gastrectomized rats. Intraperitoneal sodium pentobarbital (4.5 mg/100 g) was used for anesthesia.

Immediately following operation, each rat was given either saline or saline and tryptophan subcutaneously in the same amounts as used in the control period and the urinary N′-methylnicotinamide excretion in the following 24 hours determined. Neither water nor food was allowed during this period. At the end of the urine collection all animals were carefully autopsied. N′-methylnicotinamide was determined using the method of Huff and Perlzweig.[21]

Results. The mortality rates were rather high in all the operated animals. It was found early that the rats having their intestines removed but with the distally ligated stomach remaining, would universally develop a gastric ulcer, usually with perforation and peritonitis. 3 cc of aluminum hydroxide gel placed in the stomach by stomach tube immediately after operation, prevented the complication completely and was consequently used in all rats reported here, except of course, the gastrectomised group. Only rats surviving the 24 hour post-operative period in good condition and showing no evidence of peritonitis or hemorrhage were used.

The results in the various groups are summarized in Table I. On the basal diet alone the rats excreted 120 γ of N′-methylnicotinamide per 100 g of rat per day. 100 mg 1(—) tryptophan caused a 10-fold increase in the same unoperated rats.

The operative procedure itself caused some increase in the basal excretion level, but the magnitude of the tryptophan response was so great that this was of no consequence.

In the group having all their intestine removed, the N′-methylnicotinamide output with saline alone was 246 γ per 100 g of rat per day; with tryptophan there was a 9-fold increase, the absolute response being considerably in excess of the response to tryptophan in the control period. This, we believe, demonstrates that the intestinal bacteria are not necessary for the synthesis of nicotinic acid from tryptophan.

Several investigators[22-24] have advanced evidence that the stomach is involved in some way in the nicotinic acid deficiency state. Accordingly, it seemed appropriate to determine whether the stomach might be necessary in converting tryptophan to nicotinic acid.

Gastrectomized rats (Table I) receiving saline only, excreted 182 γ of N′-methylnicotinamide per 100 g of rat per day while those receiving tryptophan showed a 10-fold increase, indicating that the stomach was not necessary for this process.

All of the rats, both in the control and operated periods, showed wide fluctuations in their N′-methylnicotinamide output as can be seen from the ranges listed in Table I. However, the output level seemed to be rather

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[21] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1947, **167**, 157.

[22] Sydenstricker, V. P., Armstrong, E. S., Derrick, C. J., and Kemp, P. J., *Am. J. Med. Sci.*, 1936, **192**, 1.

[23] Petri, S., Norgaard, F., Trautner, K., and Klaer, W., *Acta Med. Scand.*, 1944, **117**, 90.

[24] Gillman, T., and Gillman, J. J., *J. Am. Med. Assn.*, 1945, **129**, 12.

TABLE I.
Influence of Excision of Stomach and Intestine on Synthesis of Nicotinic Acid *from* Tryptophan.

No. of rats	Wt Avg	Wt Range g	Operative procedure	Treatment	N'-methylnicotinamide output µg/100 g of rat/24 hr Avg	N'-methylnicotinamide output µg/100 g of rat/24 hr Range
26	243	170-312	None	Basal diet only	120	20-413
25	244	170-312	"	100 mg L (—) tryptophan in 10 cc saline	1274	154-2821
6	257	231-312	Intestine removed	10 cc saline	246	41-512
8	247	218-310	" "	100 mg L (—) tryptophan in 10 cc saline	2156	457-4840
6	244	217-289	Stomach removed	10 cc saline	182	22-568
6	223	170-285	" "	100 mg L (—) tryptophan in 10 cc saline	1864	682-2976

characteristic for each rat, *i.e.*, if its output was low during the control period; it would also be low in the operated period and vice versa. This correlation also held when comparing the response to tryptophan in control and operated periods. Every rat* showed a significant increase in N'-methylnicotinamide output in response to tryptophan, and the response after operation was quantitatively similar to that before operation in each individual rat.

Discussion. While the experiments reported here seem to show clearly that intestinal bacteria are not required for the synthesis of nicotinic acid from tryptophan, they cannot be interpreted to mean that under normal conditions these bacteria may not produce some of the host's supply of this vitamin. It is well known that many of the intestinal bacteria can and do synthesize nicotinic acid. It remains to be proven, however, that any of this nicotinic acid is absorbed and used by the host. The experiments of Ellinger and co-workers[1] suggest that this may occur but some of their findings have not been confirmed by others.[25]

Conclusion. Rats deprived of their intestinal bacteria showed no impairment in their ability to convert tryptophan to N'-methylnicotinamide indicating that the synthesis of nicotinic acid from tryptophan does not occur in the gastrointestinal tract.

Since this paper was prepared for publication Henderson and Hankes (PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 26) have reported a study of the conversion of tryptophan to nicotinic acid using a technic similar to that described here. The results of these two studies are in essential agreement.

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* We have observed an occasional rat in other experiments which will methylate neither nicotinic acid nor the nicotinic acid formed from tryptophan.

[25] Najjar, V. A., Holt, L. E., Jr., Johns, G. A., Mediary, G. C., and Fleischman, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 371.

17006

Inhibitory Effects of Pteroyl Glutamic Acid Preparations.

Sidney Cobb,* Olof H. Pearson, and A. Baird Hastings.
From the Department of Biological Chemistry, Harvard Medical School, Boston.

Following the suggestion of Ross *et al.*[1] that pteroyl glutamic acid (PGA)[†] might exert an inhibitory effect on the glutamic acid metabolism of brain, experiments have been performed on the effect of PGA on the respiration of brain cell suspensions *in vitro*. The experiments were originally planned to test the hypothesis that PGA may act as a metabolic competitor of glutamate or other related substrates. At first, glutamate and PGA were used in approximately equimolar concentrations, 0.005 molar. Later, PGA effects were also studied in concentrations of 0.001 molar and 0.025 molar, keeping substrate concentrations 0.005 molar.

It is not to be inferred that such high concentrations of PGA bear any relation whatever to therapeutic concentrations, but only to the information that might be gained regarding their competitive action with glutamate and other substrates.

It may be noted in this connection that the glutamate concentration in human plasma is normally about 1 mg per 100 cc,[2] whereas the PGA concentration has been reported by Denko *et al.*[3] to be only 2 μg per 100 cc. On the basis of these data, the molar concentration of PGA in plasma is about 1/1000 that of glutamate.

As will appear below, inhibiting effects when present seem to be nonspecific and by no means restricted to the inhibition of respiration when glutamate is the added substrate. Furthermore, pure pteroyl glutamic acid seems to be without inhibitory effects even at a concentration of 0.025 molar.

Methods. The oxygen consumption of cell suspensions prepared from the brains of Wistar strain white rats have been measured in the Warburg apparatus at 37°C. These were prepared in a loose fitting homogenizer of the type devised by Potter and Elvehjem[4] that was driven at 100-200 r.p.m. Eighty mg fresh weight of tissue were pipetted into each flask. The gas phase was air and the shaking rate was 105 strokes per minute with a stroke displacement of 3 cm. The suspending medium was NaCl 0.136 M, KCl 0.004 M, $MgCl_2$ 0.0005 M, and phosphate buffer 0.0075 M (pH 7.4). Calcium was deliberately omitted from the incubating medium to avoid the danger of combination with or precipitation of PGA. Its omission was found to have no demonstrable effect on the respiration results.

The substrates used were: glutamate, pyruvate, lactate, succinate, and α-ketoglutarate, each at a concentration of 0.005 molar. Through the kindness of Dr. Y. SubbaRow and his associates at the Lederle Laboratories, 5 preparations of commercial PGA, a preparation of freshly recrystallized $(PGA)_R$, and a sample of the photofission product, 2-amino-4-hydroxy-6-formylpteridine, or pteridine aldehyde, were made available to us for testing. In addition, a sample of PGA was recrystallized in our laboratory.

The solutions of substrates and of PGA were adjusted to pH 7.2-7.5 before use. The elapsed time between the sacrifice of the rat and the first readings of the manometers varied from 20-30 minutes. Manometer readings were taken at 10-15 minute intervals for

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* Fellow of the American Cancer Society under the administration of the Committee on Growth of the National Research Council.

[1] Ross, J. F., Belding, H., and Paegel, B. L., *Blood*, 1948, **3**, 68.

[†] The abbreviation PGA will be used to denote pteroyl glutamic acid preparations kindly supplied us by Lederle Laboratories. $(PGA)_R$ will be used to denote freshly recrystallized PGA.

[2] Bessman, S. P., Magnes, J., Schwerin, P., and Waelsch, H., *J. Biol. Chem.*, 1948, **175**, 817.

[3] Denko, C. W., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, **13**, 481.

[4] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

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Discussion. While the experiments reported here seem to show clearly that intestinal bacteria are not required for the synthesis of nicotinic acid from tryptophan, they cannot be interpreted to mean that under normal conditions these bacteria may not produce some of the host's supply of this vitamin. It is well known that many of the intestinal bacteria can and do synthesize nicotinic acid. It remains to be proven, however, that any of this nicotinic acid is absorbed and used by the host. The experiments of Ellinger and co-workers[1] suggest that this may occur but some of their findings have not been confirmed by others.[25]

Conclusion. Rats deprived of their intestinal bacteria showed no impairment in their ability to convert tryptophan to N′-methylnicotinamide indicating that the synthesis of nicotinic acid from tryptophan does not occur in the gastrointestinal tract.

Since this paper was prepared for publication Henderson and Hankes (PROC. SOC. EXP. BIOL. AND MED., 1949, 70, 26) have reported a study of the conversion of tryptophan to nicotinic acid using a technic similar to that described here. The results of these two studies are in essential agreement.

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* We have observed an occasional rat in other experiments which will methylate neither nicotinic acid nor the nicotinic acid formed from tryptophan.

[25] Najjar, V. A., Holt, L. E., Jr., Johns, G. A., Mediary, G. C., and Fleischman, G., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 371.

TABLE I.
Effect of PGA Preparations on Oxygen Consumption of Rat Brain Suspensions. $Q_{O_2} = \mu L\ O_2$ consumed per hour per mg dry tissue for the 60-90 minute period of observation. Concentration of all substrates = 0.005 M.

Substrate	Control Q_{O_2}	% inhibition PGA .001 M	 PGA .005 M	 PGA .025 M
Pyruvate	7.9	9	30	53
Lactate	4.7	—	—	45
Succinate	4.4	0	11	41
α-Ketoglutarate	3.8	5	13	21
Glutamate	3.8	3	16	21
None	2.3	—	—	26

TABLE II.
Comparison of the Effect of $(PGA)_R$, PGA and Pteridine Aldehyde on the Q_{O_2} of Rat Brain Suspensions for the 60-90 Minute Period. Substrate = 0.005 molar pyruvate. All experiments represent averages of duplicate or triplicate determinations. The concentrations of $(PGA)_R$ and PGA were 0.025 M; the concentration of pteridine aldehyde was 0.00026 M.

Exp. No.	Control Q_{O_2}	% inhibition $(PGA)_R$, 0.025 M	 PGA, 0.025 M
50	8.1	4	40
55	8.1	13	52
		Pteridine aldehyde	PGA, 0.025 M
61	8.8	32	—
62	7.4	38	59
63	7.7	27	47

Kalckar *et al.*[6] have found that the aldehyde photofission product of PGA, 2-amino-4-hydroxy-6-formylpteridine or pteridine aldehyde, is toxic to xanthine oxidase, xanthopterin oxidase, and quinine oxidase; and Lowry[7] has found that this substance is toxic to pteridine oxidase. Experiments were, therefore, carried out using pteridine aldehyde in a saturated solution. (This compound is only slightly soluble at pH 7.4, and in our experiments had a concentration of 5 mg per 100 cc or 0.00026 molar.) The results of these experiments are also presented in Table II. It was found that with pyruvate as substrate, the pteridine aldehyde in very low concentration inhibited the oxygen consumption of rat brain suspensions about 30%. Parallel experiments using an uncrystallized preparation of PGA caused an inhibition of about 50%. It would appear from these observations that, since the pteridine aldehyde is a breakdown product of PGA, it may well account in large measure for the inhibitory effects observed with commercial PGA preparations. Whether there are also additional inhibitory substances present must await further work.

Summary. 1. Freshly recrystallized pteroyl glutamic acid $(PGA)_R$ even in very high concentrations exerts no significant inhibitory effect on the metabolism of brain cell suspensions.

2. PGA preparations, not freshly recrystallized, have a marked inhibitory action in high concentrations (25 millimolar). In lower concentrations (1 millimolar), the inhibition is negligible.

3. The inhibitory action with pyruvate as substrate can be largely accounted for by the photofission product of PGA (2-amino-4-hydroxy-6-formyl-pteridine).

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[7] Lowry, O. H., personal communication.

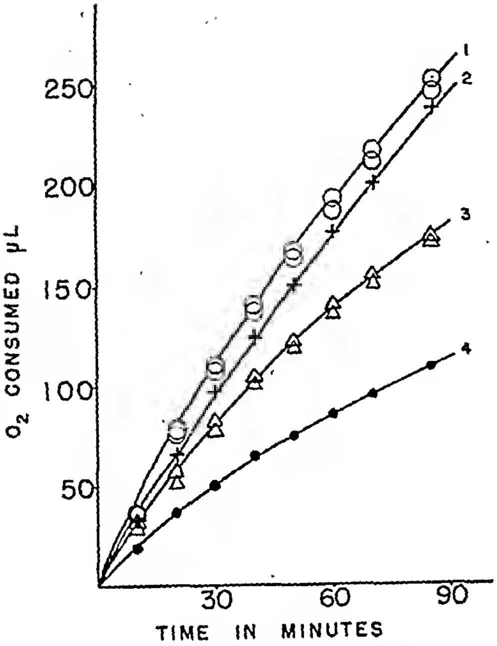

FIG. 1.

Experiment No. 50, comparison of the effect of PGA and recrystallized PGA, $(PGA)_R$, on the oxygen uptake of rat brain suspension in the presence of pyruvate. 1. Pyruvate 0.005 M with $(PGA)_R$ 0.025 M. 2. Pyruvate 0.005 M. 3. Pyruvate 0.005 M with PGA 0.025 M. 4. No substrate added. Paired circles, curve 1, and paired triangles, curve 3, denote duplicate determinations.

90 minutes and converted to microliters (µL) of oxygen consumed. Results are reported in terms of Qo_2 (µL per mg dry tissue per hour) for the 60-90 minute portion of each experiment. This period was chosen in order to increase the opportunity for any influence of added PGA preparations to become evident.

Results and Comment. The results of a typical experiment are shown in Fig. 1. This illustrates the doubling of the respiration rate produced by the presence of 0.005 molar pyruvate (compare curves 2 and 4); the absence of inhibitory effects by recrystallized pteroyl glutamate $(PGA)_R$, 0.025 molar, and the considerable inhibitory effect produced by a commercial preparation of PGA, 0.025 molar.

In Table I are presented the control Qo_2 values observed in the presence of various substrates, and the degree of inhibition produced by the addition of commercial PGA preparations in concentrations of 0.001 M, 0.005 M, and 0.025 M, respectively. It will be noted that some inhibition was produced in the presence of all substrates, or, indeed, in the absence of any added substrate, when the PGA preparation was in a concentration of 0.025 molar. The greatest inhibition was observed with pyruvate, lactate, and succinate; the least with α-ketoglutarate, glutamate, or no added substrate. No significant inhibition was observed when PGA was in a concentration of 0.001 molar; at PGA concentrations of 0.005 molar, the respiration with pyruvate as substrate was decreased 30%; with other substrates, it was not significantly altered.

In view of the absence of inhibitory effects of PGA in a concentration of 0.001 molar and in view of the non-specific nature of the inhibition encountered at PGA concentrations of 0.025 molar, it was concluded that no specific competition of PGA with glutamate was indicated by the present experiments.

Since the respiration with pyruvate seemed to be the most sensitive to additions of PGA, this substrate was used in subsequent experiments to determine (a) whether freshly recrystallized $(PGA)_R$ exerted any inhibitory effect and (b) whether breakdown products of PGA could account for the inhibition. The results of these experiments are presented in Table II.

It was found that freshly recrystallized $(PGA)_R$ exerted no significant inhibitory effect even at the high concentration of 0.025 molar, whereas experiments with commercial PGA run at the same time showed between 40 and 50 percent inhibition of respiration. From this it may be concluded that the inhibition of respiration encountered in the previous experiments was not due to the pteroyl glutamic acid present. This is in agreement with the results obtained by Franklin *et al.*[5]

Efforts were then directed to a study of the substances producing the inhibitory effect.

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[5] Franklin, A. L., Regan, M., Lewis, D., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 523.

[6] Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, 1948, **172**, 349; Kalckar, H. M., Kjeldgaard, N. V., and Klenow, H., *J. Biol. Chem.*, 1948, **174**, 771.

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[7] Lowry, O. H., personal communication.

17007 P

Serologic Relationship Between Streptococcus Group H and *Streptococcus sanguis.*

RENA L. DODD. (Introduced by Grace M. Sickles.)

From Division of Laboratories and Research, New York State Department of Health, Albany.

Definition of streptococcus group H by Hare[1] was based on cultures from apparently normal throats. Little has since been published on strains of this serologic group. Lancefield[2] included it in her review of hemolytic streptococci. Hehre and Neill,[3] studying dextran formation by streptococci from cases of subacute bacterial endocarditis, observed that many strains producing dextran belonged to group H. Loewe and others[4] described a streptococcus isolated from cases of subacute bacterial endocarditis that required large doses of penicillin for successful treatment. The strains produced green discoloration of blood agar and dextran from sucrose. They were designated *Streptococcus* s.b.e. and, later, *Streptococcus sanguis.*[5] Their relationship to group H was not determined.

Thirty-six streptococcus strains isolated from samples of air* and 4 from cases of subacute bacterial endocarditis which were identified as group H have been studied in comparison with 6 strains of group H identified by Dr. Ronald Hare, and with 2 strains of *Streptococcus sanguis,* types I and II from Dr. James Sherman.

All strains produced small colonies on blood agar and grew well in beef infusion broth.[6] Many strains produced green discoloration of blood agar. A few, including the group H strains from Doctor Hare, produced hemolytic and green colonies. All strains fermented sucrose. The colonies of all except 8 strains from air samples and 2 from subacute bacterial endocarditis were altered in appearance when grown on blood agar containing 5% sucrose.[3] Precipitation with 1.2% alcohol of the sucrose broth cultures of 27 of the strains producing such colonies suggested dextran production. No correlation was noted between dextran production and fermentation of raffinose and salicin.[3,4]

Antisera were produced in rabbits by immunization with heated and formalin treated cells of streptococcus group H No. 36251 and *Streptococcus sanguis* types I and II. Untreated hydrochloric acid extracts[7] and extracts partially purified by precipitation with three volumes of 95% alcohol[8] were used. Reproducible results were obtained with the alcohol precipitated extracts, while some variation was noted in the reactivity of the untreated ones. Extracts of the 6 representative group H strains and of the 2 strains of *Streptococcus sanguis* reacted with group H and *Streptococcus sanguis* antisera in the precipitation test. Tests with absorbed sera demonstrated antigenic differences.

Extracts of strains from air samples and from subacute bacterial endocarditis were tested with the unabsorbed sera and 3 absorbed sera (Table I). These absorbed antisera were chosen because they served to separate the group H and *Streptococcus sanguis* strains into 4 divisions represented by

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[1] Hare, Ronald, *J. Path. and Bact.*, 1935, **41**, 499.

[2] Lancefield, R. C., In Harvey Society, New York. Harvey lectures, 1940-41, Series 36, 251.

[3] Hehre, E. J., and Neill, J. M., *J. Exp. Med.*, 1946, **83**, 147.

[4] Loewe, Leo, and others, *J. Am. Med. Assn.*, 1946, **130**, 257.

[5] White, J. C., Streptococci from subacute bacterial endocarditis. Ithaca, N. Y., Cornell University, 1944, 47 p. Thesis.

* These cultures were isolated from air samples taken in schools in New York State in the course of a study of the effect of ultraviolet irradiation.

[6] Wadsworth, A. B., Standard Methods of the Division of Laboratories and Research of the New York State Department of Health: 3rd ed. Baltimore, Williams and Wilkins, 1947, p. 186.

[7] Lancefield R. C., *J. Exp. Med.*, 1928, **47**, 91.

[8] Lancefield, R. C., *J. Exp. Med.*, 1934, **59**, 441.

TABLE I.
Serologic Relationship of Representative Strains of Streptococcus Group H and *Streptococcus sanguis.*

		Antisera											
Culture extracts		Group H 36251				Sanguis type I 4647				Sanguis type II 4648			
			Absorbed with				Absorbed with				Absorbed with		
Original designation	Group and strain	Not abs.	4647	4648	36658	Not abs.	36251	4648	36658	Not abs.	36251	4647	36658
Perrier	H36251	+*	+	+	+*	+*	—	—*	—*	+*	—	+	—
Sanguis I	4647	+	—	—	—	+	+	+	+	+	—	—	—
,, II	4648	+	+	—	—	+	+	—	—	+	—	+	—
Challis	H36658	+	+	—	—	+	+	+	—	+	—	+	—

+ Indicates ring formation in 10 min. and a definite cloud or precipitate in 2 hr at 35°C.
* These sera were used in the differentiation of strains.

TABLE II.
Serologic Differences Among Strains Identified as Streptococcus Group H.

		Number of strains with reactivity similar to			
Source	Colony on 5% sucrose blood agar	No. 36251 group H	No. 4647 *Strep. sanguis* type I	No. 4648 *Strep. sanguis* type II	No. 36658 group H
Air sample	Altered	9	4	15	0
	Unchanged	2	1	5	0
Subacute bacterial endocarditis	Altered	0	0	2	0
	Unchanged	0	0	2	0

strains Nos. 36251, 4647, 4648 and 36658.

Table II indicates the distribution among these 4 divisions of strains from air and subacute bacterial endocarditis. Of 40 streptococcus strains from samples of air and from cases of subacute bacterial endocarditis identified as streptococcus group H, eleven reacted similarly to group H No. 36251; 5 appeared similar to *Streptococcus sanguis* type I and 22 to type II, under the conditions of the test. Extracts of 2 air sample strains, one of which produced colonies with altered appearance on 5% sucrose blood agar, reacted only in unabsorbed group H No. 36251 antiserum.

Summary. Representative strains of streptococcus group H and *Streptococcus sanguis* and of 40 strains from samples of air and from cases of subacute bacterial endocarditis identified as group H were tested with unabsorbed and selected absorbed antisera produced against streptococcus group H No. 36251 and *Streptococcus sanguis* types I and II. The results of the tests indicated that the strains belonged in at least 5 antigenically different types.

17008 P

Acute Disseminated Encephalomyelitis Produced in Albino Mice.

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The production in monkeys of acute disseminated encephalomyelitis accompanied by demyelination following injection of brain tissue, first achieved by Rivers, Sprunt and Berry,[1] was not only confirmed by others, but similar lesions were later obtained with various preparations in rabbits and guinea pigs. The experimental disease has not hitherto been induced in mice. It would appear that if mice so convenient for experimental work could be shown to be susceptible, a step forward might be made. As the following results will show, it is believed that this has been accomplished.

Of 40 mice W-Swiss strain,[2] in 2 series of experiments, all were injected intramuscularly with 0.3 ml of a milky suspension containing 20 mg killed tubercle bacilli, 50 ml heavy liquid petrolatum and 10 g normal mouse brain in 50 ml saline solution. The tubercle bacilli were human type, pathogenic strain H37Rv,[†] acetone-dried and autoclaved for 15 minutes at 15 lb pressure. The normal mouse brain was obtained from a healthy stock of Rockefeller Institute or W-Swiss strains of albino mice. The materials were homogenized in a small-sized Waring blendor and mice were given 3 to 6 injections of the mixture at weekly or longer intervals.

Localized nodules were induced which were absorbed with difficulty and persisted for several weeks. The nodules contained grumous material, chiefly polymorphonuclear leucocytes and epithelioid cells but no visible microorganisms.

From 16 to 105 days after the first injection, 36 mice have thus far shown definite signs of involvement of the central nervous system. They exhibited all or some of the following patterns of behavior: ruffled fur; dyspnea; paresis; paralysis, generally of the hind limbs; generalized, coarse tremors; excitation alternating with somnolence; ataxia; tip-toe gait and hunched back. Subsidence of signs and later relapse were characteristic; quiescent periods endured from 2 to 15 or more days.

The chief histological changes in the brain and cord related to the marked mural and perivascular infiltration, observed more often in the white matter. The collections of cells were made up mostly of lymphocytes and microglial cells, some polymorphonuclear leucocytes and plasma cells, monocytes and compound granular corpuscles. The infiltrations tended to spread out perivascularly into the surrounding parenchyma. Vascular leucocytic or hyaline thrombi also occurred. The meninges were free from lesions or showed a spotty similar infiltration, especially about blood vessels. Neuronal degeneration was present in scattered areas, mostly in the cord, hind brain, basal ganglia and sometimes in the Purkinje cells. Disseminated, localized and diffuse glial infiltration, and small hemorrhages were visible. Demyelination was present, but not in all animals and generally in the parenchyma but this was not a prominent lesion in these early stages of illness.

Stock mice have not shown any of these signs, and repeated examinations of their central nervous tissues failed to show any apparent lesions. In addition, 20 W-Swiss mice were injected intramuscularly with 0.3 ml of acetone-ether, lipid extract of brain obtained from apparently normal mice. The concentration of lipid was 20 mg/ml and 2 to 8 injections of an emulsion of equal parts lipid and heavy liquid petrolatum were given at intervals of not less than one week. Of these 14 were killed from 14 to 113 days after the first injection, and their brains and spinal cords revealed no visible changes. Two additional series of control tests were carried out: 1) 3 mice at the height of reaction to the induced encephalomyelitis were killed and suspensions of the brain and cord of each, in dilution of 10^{-1}, were injected intracerebrally into 10 mice. None of the 30 W-Swiss mice exhibited neurological signs. 2) The material used for inoculation was tested for the presence of an infective element transmissible to mice; none was found. It would thus appear that the induced encephalomyelitis is not caused by a transmissible agent present in the inocula or in the stock mice.

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* Lt.-Colonel, V.C., U. S. Army.

[1] Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.

[2] Webster, L. T., *J. Exp. Med.*, 1939, **70**, 87.

[†] The writers are indebted to Dr. G. Middlebrook for this strain.

To conclude, disseminated encephalomyelitis was readily induced in W-Swiss mice by means of intramuscular injections of brain tissue combined with liquid petrolatum and killed tubercle bacilli—the latter being the constitutents of a modified "adjuvant" technic of Freund and McDermott.[3]

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[3] Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, 49, 548.

17009

Tests for Chemical Mutagens in *Drosophila* Using the Vaginal Douche Technic.*†

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In a previous note[1] data were presented demonstrating that the rate of mutation is increased in spermatozoa when the latter are exposed to methyl bis (β-chloroethyl) amine hydrochloride introduced into females of *Drosophila melanogaster* by vaginal douche. In addition, there seemed to be an increase in the number of mutations occurring in the egg chromosomes of females that were treated with the N mustard in this manner. It is the purpose of this paper to offer additional data which confirms these observations and to present data on the mutagenic activity of copper sulfate, formaldehyde, 2,4-dinitrophenol, hydrazine hydrate and trypsin, employing this technic. These results will be discussed together with those of other workers testing these chemicals in *Drosophila*.

Material and method. The Oregon-R wild type and the Muller-5 (sc^{S1} B In-S w^a sc^8) stocks of *Drosophila melanogaster* were used in these experiments. Virgin females from either one of these stocks were aged for 2 to 5 days, injected with a chemical substance, and then individually placed in vials with several males from the other stock. The males had been isolated soon after hatching. Injections were made as described previously,[1] but in the later experiments a set-up of ringstand clamps, arranged to steady the hands during injection, was substituted for the micromanipulator. Females were observed for copulation for 2 hours after the injection. Only a single mating was permitted, after which the males were killed and the females placed individually in "creamers" or vials containing culture medium. Transfers to fresh creamers or vials were made every 2 to 3 days until no further offspring were produced. As in the earlier experiments, all the first generation females except those lost through accident were tested for lethal mutations involving the X chromosome from the Ore-R stock. Except for a period in the N mustard experiments reported here, in which the food was not enriched with brewer's yeast, all the first generation females were also tested for lethals involving the X chromosome from the Muller-5 stock. The plan of the crosses made for

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* Part of this investigation was done at Columbia University, New York, and the Long Island Biological Laboratory at Cold Spring Harbor, N. Y.

† This work has been supported by a grant from the U. S. Public Health Service.

[1] Herskowitz, I. H., *Evolution*, 1947, 1, 111.

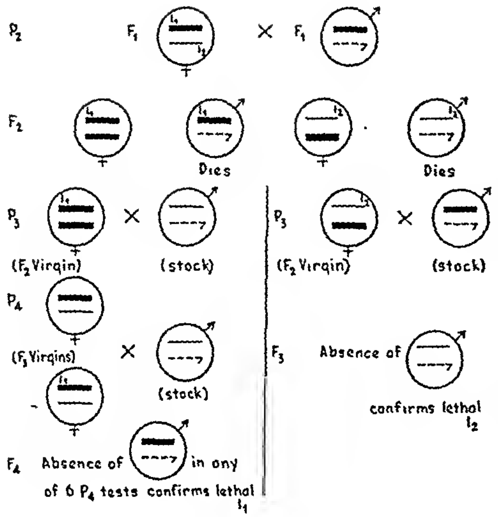

FIG. 1.

Plan of crosses to detect and confirm lethals in either X chromosome of F_1 ♀♀ from crosses between Ore-R and Muller-5 stocks. Heavy line = X chromosome from one of these stocks; light line = X chromosome from the other; broken line = Y chromosome. Single pair matings throughout.

the detection and confirmation of lethals involving either of the X chromosomes of F_1 females is presented in Fig. *1* where it is assumed for clarity that both X chromosomes have different lethal mutations (l_1 and l_2). Lethal mutations originating in the sperm X chromosome (l_2) can be detected in the F_2 and retested in the F_3 generation. Lethals originating in the egg X chromosome (l_1) also can be detected in the F_2 but require an F_4 generation for confirmation.

In the N mustard experiments females failing to copulate within the 2 hour period after injection were isolated individually with one male for an unlimited period of time. The rest of the procedure was the same for these females as for those that had copulated within the time allotted, with one exception. Instead of using all the first generation females, only random samples from the first vial and from the transfer vials yielding the last female progeny were taken for tests of lethals.

F_2 cultures containing potential lethals were counted every 2 to 3 days for a month after the cross was made or until no further offspring emerged. If few or no males appeared of either the Ore-R or Muller-5 type the appropriate X chromosome was retested in a subsequent generation. Retests for mutations were performed under the same experimental conditions used for obtaining the F_2 generation. A lethal mutation was considered confirmed if the total retest population contained 1% or less of males with the tested X chromosome, and a semilethal confirmed if 1-10% of the individuals in the retest generation were males with the X chromosome tested.

Saline, prepared according to Buck and Melland,[2] was the solvent for all the chemical substances except formaldehyde, which was prepared with distilled water, and hydrazine hydrate which was used undiluted. The solutions were made just prior to use. Cultures were kept at room temperature.

Results. The fate of 414 females treated with chemicals by vaginal douche is presented in Table I. Of these, 294 females (71%) copulated within 2 hours after injection. However, this figure is composed of only 20 of the 66 Ore-R females treated (30%) as compared with 274 of the 348 Muller-5 females (79%). In a total of 359 copulations observed with Muller-5 females in these and similar experiments, 47 (13%) occurred while the females were anesthetized; among the 20 copulations observed with Ore-R females none occurred while the females were still under the effects of ether. Copulation with etherized females occurred in a variety of experiments involving the injection of 7 different chemical substances and resulted in apparently normal deliveries of sperm. The concentration of the chemical substance injected seems to affect the number of females that oviposit and, of these, the number that produce adult offspring. When the highest concentrations of copper sulfate and formaldehyde were used only 10 of 54 females that copulated (19%) deposited eggs. When intermediate concentrations of these chemicals were injected 20 of 34 females oviposited (60%), and with the lowest doses 28 of 42 (67%). With the highest concentrations of copper sulfate and formaldehyde none of the 10 ovipositing females produced adult pro-

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[2] Buck, J. B., and Melland, A. M., *J. Hered.*, 1942, **33**, 173.

TABLE I.
Fate of ♀♀ Treated with Chemicals by Vaginal Douche.

Chemical	Conc., %	Date	Stock	No. ♀♀ Injected	No. ♀♀ Copulating within 2 hr	No. ♀♀ Ovipositing sterile eggs	No. ♀♀ Producing adult offspring
N mustard	4	9/48	M-5	71	55	9	26
			Ore-R	50	12	—	8
Formaldehyde	10	9/47	M-5	19	18	2	0
	5		M-5	28	22	3	13
	1		M-5	19	14	1	12
Copper sulfate	saturated sol. and 5%	9/47	M-5	46	36	7	0 (1 larvae)
	1.0		M-5	15	12	3	1
	0.2		M-5	34	28	5	9 ,,
2,4-Dinitrophenol	saturated sol.	5/47	M-5	78	53	2	32
		7/47	Ore-R	16	8	1	6
Hydrazine hydrate	85	7/47	M-5	25	23	1	16
Trypsin	conc. sol.	7/47	M-5	13	13	0	8

TABLE II.
Summary of Tests for Mutagenic Action of Several Chemical Substances.*

		X chromosome lethals†				
		Muller-5 ♀♀ injected		Oregon-R ♀♀ injected		
Chemical	Conc., %	No. (Ore-R) lethals/ No. sperm tested	No. (M-5) lethals/ No. sperm tested	No. (M-5) lethals/ No. sperm tested	No. (Ore-R) lethals/ No. eggs tested	Total lethals/ total gametes tested
N mustard	4	16/1043	6/710	7/373	3/373	32/2499
Formaldehyde	1 and 5	1/549	0/547			1/1096
Copper sulfate	0.2 and 1.0	0/183	1/150			1/333
2,4-Dinitrophenol	saturated sol.	1/751	2/739	0/562	1/562	4/2614
Hydrazine hydrate	85	1/623	2/593			3/1216
Trypsin	conc. sol.	2/506	2/506			4/1012

* Only includes tests from ♀♀ copulating within 2 hr after injection.
† All lethals were confirmed.

geny; however, when lower concentrations of these chemicals were used 35 of the 48 females that oviposited (73%) produced adult offspring.

The results of the tests for X chromosome lethals, in the daughters of females that copulated within 2 hours after injection with different chemical substances, are summarized in Table II. Only 13 lethals were observed in the 6271 sperm or egg X chromosomes tested in the formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin experiments. However, the N mustard treatments produced 32 lethals in 2499 sperm or egg X chromosomes tested, of which 23 lethals from 11 origins occurred in 1416 sperm chromosomes tested and 9 lethals from 8 origins occurred in 1083 egg chromosomes tested.

In the N mustard experiments females failing to copulate within the time allotted were not discarded. Instead, these females were utilized to obtain offspring that were the issue of the single pair matings taking place sometime later than 2 hours after injection. The results of egg and sperm X chromosome lethal

TABLE III.
The X Chromosome Lethal Mutation Rate in ♀♀ Copulating Later than 2 Hours After Injection with 4% N Mustard.*

Stock ♀♀ injected	No. fecund/ No. treated	No. (Ore-R) lethals/ No. sperm tested	No. (M-5) lethals/ No. eggs tested	No. (M-5) lethals/ No. sperm tested	No. (Ore-R) lethals/ No. eggs tested
Muller-5	7/16	1/444	4/363	—	—
Oregon-R	23/38	—	—	6/1091	10/1091

* All lethals were confirmed except 2 lost.

TABLE IV.
The Distribution, in the 4% N Mustard Experiments, of F_1 ♀♀ Bearing Lethals in Relation to the Total Number Tested.

Transfer vial No.	0	1	2	3	4	5	6 and 7
No. ♀♀ with sperm lethals/ No. ♀♀ tested	9/649	6/601	3/366	3/382	2/309	5/511	2/133
No. ♀♀ with egg lethals/ No. ♀♀ tested	18/649	1/564	1/188	1/268	1/246	1/489	0/133

tests with such female progeny are presented in Table III. Twenty-one lethals were obtained in a total of 2989 egg and sperm X chromosomes tested. However, only 7 of these lethals (from 6 origins) occurred in the 1535 sperm tested, whereas 14 lethals (from 13 origins) arose in the 1454 eggs tested.

Table IV shows the distribution in the various transfer vials of lethal bearing F_1 females in relation to the total number of females tested. Transfers were made every 2 days. The data come from parents copulating before as well as after the 2 hour period following injection with 4% N mustard. The 30 sperm lethals in the 2951 sperm chromosomes tested seem to be distributed at random in the transfer vials. This is not the case for the 23 egg lethals in the 2537 egg chromosomes tested. The frequency of egg lethals in F_1 females oviposited during the first two days (18/649) is significantly larger ($P < .01$) than the frequency of egg lethals in females coming from eggs deposited after that time (5/1888).

Besides the lethals, 2 visible mutants (rough and white eyes) and 2 semi-lethals occurred in the 4% N mustard experiments. These mutants occurred in the sperm X chromosomes of progeny from 3 females which had copulated within the two hours allotted. In addition, each of these parents had produced sperm lethals.

Discussion. The data in Table I offer several points of interest concerning the use and effects of vaginal douches in relation to studies of chemical mutagens in *Drosophila*. Much stronger concentrations of chemical substances may be used in vaginal douche treatments than in most other technics designed to treat gametes with chemicals for the purpose of inducing mutations. This seems to be due to the fact that only a portion of the individual is treated with the chemical substance in this procedure. Low concentrations of mutagens may be effective because of the direct contact of the chemical with the sperm.[1] However, very strong concentrations of chemical substances can produce definite harmful effects on the females treated. Failure of injected females to oviposit is due in part to the fact that many females are weakened by the chemical and die in the first few days after the treatment. This is reflected in the decrease in the percent of females which oviposit after copulation as the concentrations of copper sulfate and formaldehyde are increased. The evidence that the percent of ovipositing females producing adult progeny is lower with the highest concentrations of copper sulfate and formaldehyde than with more moderate concentrations has several possible interpretations. High concentrations of these chemicals may cause many gametes to become non-functional and/or may produce genetic or physiological changes subsequent to fertilization that result in death before maturity. Such effects should also result in a decrease in the number of adult offspring produced when the concentration of the chemical substance injected is increased. The vaginal douche experiments that employed 0.2% and 10.0% N mustard[1] were performed under comparable experimental conditions and furnish information on this point. Unfortunately, data from the experiments with 4% N mustard are not suitable for comparison since the food used had been enriched with brewer's yeast. The average number of males and females in the F_1 progeny per fecund female was 74 in the 0.2% treatments (from 24 parents) but was only 29 (from 9 parents) in the 10.0% treatments.

In the formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin experiments (Table II) there were no lethals in 562 Muller-5 sperm X chromosomes tested and 7 lethals in 2535 Muller-5 egg X chromosomes tested. In the same experiments there were 5 lethals in 2612 Ore-R sperm X chromosomes tested and one lethal in 562 Ore-R egg X chromosomes tested. This suggests that the spontaneous lethal mutation rate in the Muller-5 X chromosome is much the same as in the Ore-R.[3-5] Under comparable experimental conditions we may also note the mutational response of these two chromosomes when exposed to the action of a mutagenic agent (Table II). In treatments with 4% N mustard 16 lethals occurred in 1043 Ore-R sperm tested and 7 in 373 Muller-5 sperm tested; 3 lethals occurred in 373 Ore-R eggs tested and 6 in 710 Muller-5 eggs tested. These data suggest that both the Oregon-R and the Muller-5 X chromosomes mutate with similar frequencies when exposed to N mustard. Accordingly, no distinction will be made between Ore-R and Muller-5 X chromosomes in the discussion which follows.

Vaginal douches with India ink suspensions showed that particles of the ink remained in the vagina of some virgins when these were dissected 12 hours after injection.[1] It seemed desirable, nevertheless, to test the egg and sperm lethal mutation rate in females copulating later than 2 hours after vaginal douche with 4% N mustard solution. In these experiments (Table III), there were 7 lethals in 1535 sperm chromosomes tested while in a similar number of egg chromosomes tested, 1454, there were 14 lethals. While the egg mutation rate in these experiments (14/1454) and the mutation rate in eggs from females copulating within 2 hours after injection (9/1083, Table II) seem to be comparable, the sperm mutation rate (7/1535) is considerably lower than the one in sperm deposited soon after injection (23/1416, Table II). This effect may be attributed to the removal of the mutagenic agent from the vagina sometime after the two hours allotted for copulation. The removal of the mutagenic agent may have been the result of any one or more of the following: diffusion through the body, expulsion from the vagina, chemical decay of the agent, and chemical combination with the body substances.

In the 4% N mustard treatments the lethal mutation rate in egg X chromosomes (23/2537, Tables II and III) appears to be lower than the rate in sperm X chromosomes from females copulating soon after injection (23/1416, Table II). An examination of the distribution of the egg and sperm lethals from the 4% N mustard experiments in relation to the time of hatching of the F_1 females may lead to a better understanding of the basis for the apparent difference in mutability of egg and sperm X chromosomes (Table IV). The distribution of sperm lethals in the various transfer vials seems to be at random. Accepting the hypothesis that the mutagenic agent disappears from the vagina soon after injection, this distribution is consistent with the

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[3] Demerec, M., *Carnegie Inst. Wash. Yearb.*, 1946, **45**, 156.

[4] ———, *Nature*, 1947, **159**, 604.

[5] ———, *Genetics*, 1948, **33**, 337.

TABLE III.
The X Chromosome Lethal Mutation Rate in ♀♀ Copulating Later than 2 Hours After Injection with 4% N Mustard.*

Stock ♀♀ injected	No. fecund/ No. treated	No. (Ore-R) lethals/ No. sperm tested	No. (M-5) lethals/ No. eggs tested	No. (M-5) lethals/ No. sperm tested	No. (Ore-R) lethals/ No. eggs tested
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Discussion. The data in Table I offer several points of interest concerning the use and effects of vaginal douches in relation to studies of chemical mutagens in *Drosophila*. Much stronger concentrations of chemical substances may be used in vaginal douche treatments than in most other technics designed to treat gametes with chemicals for the purpose of inducing mutations. This seems to be due to the fact that only a portion of the individual is treated with the chemical substance in this procedure. Low concentrations of mutagens may be effective because of the direct contact of the chemical with the sperm.[1] However, very strong concentrations of chemical substances can produce definite harmful effects on the females treated. Failure of injected females to oviposit is due in part to the fact that many females are weakened by the chemical and die in the first few days after the treatment. This is reflected in the decrease in the percent of females which oviposit after copulation as the concentrations of copper sulfate and formaldehyde are increased. The evidence that the percent of ovipositing females producing adult progeny is lower with the highest concentrations of copper sulfate and formaldehyde than with more moderate concentrations has several possible interpretations. High concentrations of these chemicals may cause many gametes to become non-functional and/or may produce genetic or physiological changes subsequent to fertilization that result in death before maturity. Such effects should also result in a decrease in the number of adult offspring produced when the concentration of the chemical substance injected is increased. The vaginal douche experiments that employed 0.2% and 10.0% N mustard[1] were performed under comparable experimental conditions and furnish information on this point. Unfortunately, data from the experiments with 4% N mustard are not suitable for comparison since the food used had been enriched with brewer's yeast. The average number of males and females in the F_1 progeny per fecund female was 74 in the 0.2% treatments (from 24 parents) but was only 29 (from 9 parents) in the 10.0% treatments.

In the formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin experiments (Table II) there were no lethals in 562 Muller-5 sperm X chromosomes tested and 7 lethals in 2535 Muller-5 egg X chromosomes tested. In the same experiments there were 5 lethals in 2612 Ore-R sperm X chromosomes tested and one lethal in 562 Ore-R egg X chromosomes tested. This suggests that the spontaneous lethal mutation rate in the Muller-5 X chromosome is much the same as in the Ore-R.[3-5] Under comparable experimental conditions we may also note the mutational response of these two chromosomes when exposed to the action of a mutagenic agent (Table II). In treatments with 4% N mustard 16 lethals occurred in 1043 Ore-R sperm tested and 7 in 373 Muller-5 sperm tested; 3 lethals occurred in 373 Ore-R eggs tested and 6 in 710 Muller-5 eggs tested. These data suggest that both the Oregon-R and the Muller-5 X chromosomes mutate with similar frequencies when exposed to N mustard. Accordingly, no distinction will be made between Ore-R and Muller-5 X chromosomes in the discussion which follows.

Vaginal douches with India ink suspensions showed that particles of the ink remained in the vagina of some virgins when these were dissected 12 hours after injection.[1] It seemed desirable, nevertheless, to test the egg and sperm lethal mutation rate in females copulating later than 2 hours after vaginal douche with 4% N mustard solution. In these experiments (Table III), there were 7 lethals in 1535 sperm chromosomes tested while in a similar number of egg chromosomes tested, 1454, there were 14 lethals. While the egg mutation rate in these experiments (14/1454) and the mutation rate in eggs from females copulating within 2 hours after injection (9/1083, Table II) seem to be comparable, the sperm mutation rate (7/1535) is considerably lower than the one in sperm deposited soon after injection (23/1416, Table II). This effect may be attributed to the removal of the mutagenic agent from the vagina sometime after the two hours allotted for copulation. The removal of the mutagenic agent may have been the result of any one or more of the following: diffusion through the body, expulsion from the vagina, chemical decay of the agent, and chemical combination with the body substances.

In the 4% N mustard treatments the lethal mutation rate in egg X chromosomes (23/2537, Tables II and III) appears to be lower than the rate in sperm X chromosomes from females copulating soon after injection (23/1416, Table II). An examination of the distribution of the egg and sperm lethals from the 4% N mustard experiments in relation to the time of hatching of the F_1 females may lead to a better understanding of the basis for the apparent difference in mutability of egg and sperm X chromosomes (Table IV). The distribution of sperm lethals in the various transfer vials seems to be at random. Accepting the hypothesis that the mutagenic agent disappears from the vagina soon after injection, this distribution is consistent with the

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[3] Demerec, M., *Carnegie Inst. Wash. Yearb.*, 1946, **45**, 156.

[4] ———, *Nature*, 1947, **159**, 604.

[5] ———, *Genetics*, 1948, **33**, 337.

evidence that the genes in mature sperm are not functional.[6,7] The preponderant occurrence of egg lethals in the individuals oviposited during the first 2 days may have several explanations. Since, in all the experiments with N mustard there were no F_1 females with lethals on both X chromosomes, and in view of the data indicating that egg chromosomes mutate when sperm chromosomes do not (Table III), it is suggested that the production of egg mutations is independent of the treatment of sperm and that eggs are treated with the mutagenic agent directly. However, the means whereby eggs are exposed to the mutagenic agent still remains obscure and the apparent difference in the mutability of eggs and sperm may be the result of differences in the concentration of mutagen to which each is exposed or to a difference in the mutability of mature and immature ova.

We shall consider briefly the results of lethal tests from the N mustard, copper sulfate, formaldehyde, and 2,4-dinitrophenol experiments (Table II) with reference to the studies of other workers employing these and related chemicals in *Drosophila*. The pioneer work of Auerbach and Robson[8-10] clearly demonstrated that mustard gas and certain N and S mustards are chemical mutagens. Using the vapor and spray technics to treat mature sperm, they obtained up to 24% lethals with mustard gas and up to 13% with N mustard. Demerec[3,4] found the N mustard, methyl bis (β-chloroethyl) amine hydrochloride, to be mutagenic in treatments of spermatocytes and mature sperm using the aerosol technic. The author, in this and a previous paper,[1] has presented evidence that mutations in eggs and mature sperm are produced when this N mustard is used in vaginal douches. Such factors as the solvent for, and the concentration of, the chemical substance used should be taken into consideration in evaluating different technics for the chemical induction of mutations.

Magrizhikovskaja,[11] by bathing whole eggs, Law,[12] by injecting larvae and bathing dechorionated eggs, and Zamehof,[13] by feeding larvae, all obtained data indicating that copper sulfate is mutagenic. However, Demerec,[3] using aerosols to treat mature sperm and spermatocytes, and the author, using vaginal douches to treat eggs and mature sperm, obtained negative results with this chemical. Rapoport[14] and Kaplan[15] found positive mutagenic effects after feeding early developmental stages with formaldehyde but the author, using vaginal douches, obtained negative results. Thornton[16] has shown that 2,4-dinitrophenol decreases the growth rate and delays the moulting and pupation of *Drosophila* larvae. This drug is known to speed up the intracellular oxidative metabolism, principally of fat, in rats[17] and to inhibit phosphate uptake in staphylococci and yeast.[18] Assuming the effects of this chemical are similar in *Drosophila* it seemed desirable to test whether this chemical could influence the mutation rate via some metabolic effect. Vaginal douches with solutions of this chemical have not produced a detectable increase in the lethal mutation rate of sperm or egg X chromosomes.

Although negative results, in general, may be due to a variety of factors, it may be of interest to suggest some reasons for the failure of the aerosol and vaginal douche technics to produce mutations after treatments with copper sulfate and formaldehyde. If these

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[6] Muller, H. J., and Settles, F., *Z. f. ind. Abst.-u. Verer.*, 1927, **43**, 285.

[7] Dobzhansky, Th., *Genetics*, 1930, **15**, 347.

[8] Auerbach, C., and Robson, J. M., *Nature*, 1946, **157**, 302.

[9] ———, *Proc. Roy. Soc. Edin.*, Sec. B, 1947, **62**, 271.

[10] ———, *Proc. Roy. Soc. Edin.*, Sec. B, 1947, **62**, 284.

[11] Magrzhikovskaja, K. W., *Biol. Zh.*, 1938, **7**, 635.

[12] Law, L. W., *Proc. Nat. Acad. Sci.*, 1938, **24**, 546.

[13] Zamenhof, S., *J. Genet.*, 1945, **47**, 69.

[14] Rapoport, I. A., (Doklady), *C.R. Acad. Sci. U.R.S.S.*, 1947, **56**, 537.

[15] Kaplan, W. D., *Science*, 1948, **108**, 43.

[16] Thornton, D., *Growth*, 1947, **11**, 51.

[17] Hall, V. E., Field, J., Sahyun, M., Cutting, W. C., and Tainter, M. L., *Am. J. Physiol.*, 1933, **106**, 432.

[18] Hotchkiss, R. D., *Advances in Enzymology*, 1944, **4**, 153. Interscience Pub., Inc., New York.

chemicals do reach and affect eggs, spermatocytes and mature sperm, and some support for this has been presented, the negative results obtained with these technics may be due to the relatively short duration of the treatments or to differences in the mutability of the germ plasm at various stages of its differentiation.

Summary. Vaginal douches of 4% methyl bis (β-chloroethyl) amine hydrochloride produced 53 lethals in 5488 egg and sperm X chromosomes tested while only 13 lethals were observed in the 6271 sperm or egg X chromosomes tested after treatments with formaldehyde, copper sulfate, 2,4-dinitrophenol, hydrazine hydrate and trypsin. The practicality of the vaginal douche technic for the induction of mutations with N mustard in both sperm and eggs, suggested from previous experiments, is confirmed in that 30 lethals occurred in the 2951 sperm tested and 23 in the 2537 eggs tested. The number of sperm X chromosome lethals seems to be considerably higher in F_1 females resulting from single copulations within a two hour period from the time of injection (23 lethals in 1416 tests) than it is in females from copulations after this interval (7 lethals in 1535 tests); the mutation rate for the egg X chromosome under these conditions remains apparently unaffected (9/1083 and 14/1454, respectively). Whereas the 30 sperm lethals seem to be distributed at random among the F_1 females oviposited at various times, the frequency of egg lethals in individuals oviposited during the first two days (18/649) is statistically larger than the frequency of lethals in F_1 females coming from eggs deposited after that time (5/1888).

The use and effects of vaginal douche treatments are discussed and results of the experiments evaluated in the light of studies of other workers testing these chemicals in Drosophila.

I wish to express my sincere appreciation to Dr. Walter J. Burdette for many helpful suggestions concerning the manuscript and to Professor Th. Dobzhansky for numerous discussions and suggestions throughout the course of these investigations.

17010

Effect of Alloxan Diabetes on Fertility and Gestation in the Rat.*

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The discovery of the diabetogenic properties of alloxan[1] gave to the study of metabolism a tool by means of which many vexing problems may be investigated. One of these is the effect of diabetes on reproduction, a problem whose importance has been emphasized by White and Hunt[2] in extensive investigations on human diabetics. Reproduction in animals made diabetic with alloxan has been studied by Miller,[3] Friedgood and Miller,[4] Davis, Fugo and Lawrence[5] and Hultquist.[6] Reported below are some observations on the rat made diabetic with alloxan in which the problem of fertility, gestation and the via-

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* From the thesis presented to the Graduate School of the University of Colorado by Jo Anne Sinden in partial fulfillment of the requirements for the M.S. degree.

[1] Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

[2] White, P., and Hunt, H., *J. Clin. Endocrinology*, 1943, **3**, 500.

[3] Miller, H. C., *Endocrinology*, 1947, **40**, 251.

[4] Friedgood, C. E., and Miller, A. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 61.

[5] Davis, M. E., Fugo, N. W., and Lawrence, K. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 638.

[6] Hultquist, G., *Acta Path. et Microbiol. Scand.*, 1948, **25**, 131.

TABLE I.
Reproduction Record of Untreated Diabetic Animals.

	Blood sugar, mg per 100 ml				No. of offspring		
Rat No.	Before breeding	During gestation	Post-partum	Gestation period (days)	Living	Dead	Avg body wt of offspring, g
18	398	366		22	8		5.2
29	354	328	422	23	9		5.3
30	460	396	468	23	8	2	4.8
48	372	236	452	23	9	1	5.7

TABLE II.
Reproduction Record of Insulin-Treated Diabetic Rats.

	Blood sugar				No. of offspring per litter		
Rat No.	Before insulin	With insulin	Daily insulin units	Gestation period (days)	Living	Dead	Avg body wt of offspring, g
24	549	280	7	22	7		4.93
29	422	236	9	23	10		5.50
30	468	228	9	22	6	1	4.79
56	474	186	7	23	8		4.75
62	476	137	9	24	4	1	5.07

bility of the fetus were investigated.

Experimental. Young female white rats, 90 to 120 days of age, from our own colony were used. The animals were kept in individual cages and were fed Purina Laboratory Chow. Lettuce was given once a week. Alloxan was administered intravenously via a tail vein in the amount of 55 mg per kilo of body weight. The method was essentially that of Lazarow and Palay.[7] The success of the production of diabetes was ascertained by the determination of the sugar content of blood obtained from the tip of the tail. A micro-modification of the method of Folin and Wu[8] was used for this determination.

The estrous cycle was followed by the vaginal smear method. When the animals were found to be in estrus, they were placed overnight with a young male of known fertility. On the following day, which was counted as the first day of gestation, the female was isolated. When the young were born they were counted and weighed within approximately 8 hours (maximum time). In all groups they were left with the mother so that her success in rearing the young might be observed.

Four groups of animals were studied. The first group received no treatment of any kind and served to establish the reproductive pattern of this strain of rats. Alloxan diabetes was produced in the animals of the second group, after which their ability to reproduce was studied without further treatment. The diabetic animals of the third group were given regular insulin twice daily with frequent determinations of the blood sugar until the estrous cycle returned. The time required for the resumption of estrus varied from 9 to 40 days. They were placed with the male at that time. The fourth group consisted of those animals which began pregnancy as diabetics and which demonstrated subsidence of hyperglycemia during the course of gestation.

Results. Normal Control Group. Fifteen rats were used as normal controls. The estrous cycle was 4 to 5 days in length. Fourteen had successful pregnancies, but 3 had to be placed with the male twice, 1 three times and 1 six times before they conceived. The remainder conceived at the first mating. The length of the gestation period was 22 to 23 days. The litters ranged from 4 to 13 in number and the weight of the newborn animals (average weight of the young in each litter) varied from 4.65 to 6.6 g.

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[7] Lazarow, A., and Palay, S. L., *J. Lab. and Clin. Med.*, 1946, **31**, 1004.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

Untreated Diabetic Group. The animals in this group were treated with alloxan and the vaginal smears were followed daily thereafter. Estrus appeared at intervals of 3 to 8 days following the alloxan administration. There were 13 diabetic animals in this group of which 9 failed to reproduce. The placental sign was observed in 3 of the latter 9 rats and a vaginal plug was seen in 4 of the 9 after they were with the males. In these animals the blood sugar values ranged between 365 and 468 mg per 100 ml.

The data concerning the 4 rats which did bear litters are given in Table I. Rat 18 killed and ate her young soon after parturition. The young from rat 30 died on the fourth day after parturition. Rat 29 cared for her young, but on the 14th postpartum day it became evident that she was unable to nourish them adequately and they were placed with a foster mother. They quickly gained weight and were successfully reared to the weaning age. Rat 48 reared all of her 9 live young to the weaning age.

Insulin Treated Diabetic Group. These animals were treated with regular insulin until a definite decrease in blood sugar had been established. With adequate treatment the estrous cycle was resumed in 5 of 7 animals even though the blood sugar values had not reached a normal level. The results of the reproduction experiment with this group are recorded in Table II. Of the 5 which resumed estrus, every one conceived and bore a viable litter. The young of rat 24 lived only 2 days. Rat 62 bore 4 live and 1 dead offspring, and the 4 live ones died within 3 days.

Rats 29 and 30 had borne litters while diabetic and untreated (Table I). Their estrous cycles were not resumed after the birth of their young until insulin treatment was begun. They then conceived and bore litters. Rat 29 bore 10 live rats which were reared to the weaning age and 6 living and 1 dead young were born to rat 30. The living young died within 3 days.

Rat 56 bore 8 living offspring which were accidentally sacrificed soon after birth.

Untreated Group with Transitory Diabetes. One group of 5 animals had only mild or transitory diabetes. Indeed, in 2 of these animals the blood sugar never did reach a level which would justifiably place them in the diabetic group. However, they were bred and produced litters, and the blood sugar decreased during the course of pregnancy to normal levels. The number of rats per litter varied from 5 to 13 and the average weight of the young varied between 4.6 and 6.1 g. The litter of one of these rats was born dead. These results seem to indicate that the administration of alloxan does not interfere with the ability to reproduce when it has been unsuccessful in the establishment of diabetes.

Discussion. The most recent report on this problem is that of Hultquist.[6] He produced diabetes both by pancreatectomy and by alloxan administration. Most of his animals were made diabetic after pregnancy was established and "only in a few instances" did he observe pregnancy in an already diabetic animal. He also observed that insulin improved the chances of the mother to bear young and that only one of many animals completed gestation without insulin treatment. The results herein reported show a higher percentage of successful pregnancies in untreated diabetic animals than his report seems to indicate, but also, marked improvement was observed when insulin was given, even though, again in agreement with Hultquist, the blood sugar was not regulated to non-diabetic values.

The experiments of Davis, Fugo and Lawrence[5] vary somewhat from the present ones. They observed a prolongation of the sex cycle from 4 to 5 days to 9 to 12 days with irregularity, in contrast to the observations reported above which showed one, or at the most, 2 estrous periods following a diabetogenic dose of alloxan, after which the cycle was not restored unless insulin was administered. Furthermore, none of their diabetic animals which were bred after becoming diabetic produced litters, in contrast to the 4 among 13 rats which accomplished successful gestation in the present experiments. The smears were not examined for spermatozoa in our untreated diabetic rats after they had been with the male, and it is possible that some of the 9 animals which did not conceive failed of successful coitus.

When diabetic animals were treated with insulin their cycles returned and they were successfully bred and produced litters, in agreement with the findings of Davis, Fugo and Lawrence. The young either were not in good health or the mother's milk supply was inadequate as evidenced by the early death of all but one of the litters. Also confirming the findings of these authors, we observed that those animals which recovered spontaneously from hyperglycemia were able to reproduce. Alloxan, *per se*, in the doses used did not interfere with the ability of these animals to reproduce.

Miller[3] reported a successful gestation in 3 diabetic rats which received no insulin. They were only mildly diabetic as judged by the mild glycosuria. Friedgood and Miller[4] gave alloxan to pregnant rats on the fourteenth day of gestation. They determined that the blood of the fetuses was hyperglycemic at a level only slightly below that of the mother, and that the blood sugar of the offspring had returned to normal within one day. In the present experiments it was observed that the young of the 2 surviving litters from diabetic mothers had blood sugar values within the normal range. These determinations were performed after the young had been weaned. In consideration of these findings and the report of Dohan and Lukens[9] that short exposure of cats to hyperglycemia will produce diabetes, the resistance of the fetus of the rat to maternal hyperglycemia needs further investigation.

Abnormally large body weight is commonly observed in babies borne by human diabetics.[10] The young rats in this experiment, both from treated and untreated diabetic mothers did not exceed in weight those from non-diabetic mothers. In fact, the highest average weight in the litters occurred in the offspring of one of the mothers of the non-diabetic group.

The existence of diabetes in the mother, whether treated or untreated, did not affect the length of the gestation period significantly.

Summary. Rats were made diabetic with alloxan and their ability to reproduce was studied under various conditions. The following observations were made: (1) the estrous cycle ceased in diabetic females and was restored by the administration of insulin; (2) 4 of 13 untreated diabetic animals, which were bred before the estrous cycle ceased, produced litters; (3) insulin treated diabetic animals reproduced in spite of a continuing hyperglycemia; (4) transient hyperglycemia did not interfere with reproduction and (5) diabetes in the mother did not result in hyperglycemia in the offspring in the few animals tested.

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[9] Dohan, F. C., and Lukens, F. W. D., *Endocrinology*, 1948, **42**, 244.

[10] Joslin, E. P., Root, H. F., White, P., Marble, A., and Bailey, C. C., The Treatment of Diabetes Mellitus, Lea and Febiger, Philadelphia, 1946, 773.

17011

Effect of Succinylsulfathiazole on the Urinary Excretion of Folic Acid by the Rabbit.*

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Previous studies[1] showed that the growing rabbit does not require a dietary source of pantothenic acid or riboflavin when a purified diet is fed. Further, the urinary and fecal excretion of these vitamins and also of biotin greatly exceeded the intake and was not appreciably reduced when 1% of succinylsulfathiazole was included in the diet. Preliminary experiments indicated that the folic acid excretion was greatly reduced when the sulfa drug was fed; consequently these studies have been extended to obtain further information on the excretion of folic acid by rabbits receiving purified diets with and without the addition of succinylsulfathiazole.

Experimental. In the first experiment New Zealand white rabbits with an initial weight of approximately 1060 g were fed the basal ration or the basal ration plus 2% succinylsulfathiazole. Six rabbits were used in each group. The basal ration was composed of corn oil 8%, cellulose 12%, Salts IV[2] 4%, vitamins A and D concentrate 0.5%, cerelose to 100% and tocopherols, 2-methyl 1-4-naphthoquinone, thiamine, riboflavin, nicotinic acid, pyridoxine, Ca pantothenate, choline, inositol, biotin and para-aminobenzoic acid in adequate amounts.[1] After 3-4 weeks on experiment quantitative urine collections were made and the amount of folic acid in the urine was determined with pteroylglutamic acid as the standard and *S. faecalis* R the test organism.[3] Individual urine collections were made for 3 day periods from the 4th to the 10th week alternately for 3 of the 6 rabbits in each group. The technics used for the urine collections and preservation were described previously.[1] Other groups fed the basal ration + 2.0 mg of pteroylglutamic acid per kg of ration and the basal ration + pteroylglutamic acid and succinylsulfathiazole were included in this experiment, however the urinary excretion of folic acid was not determined for these groups.

A second experiment was carried out with 8 rabbits fed the basal ration and 8 fed the basal ration plus succinylsulfathiazole. The initial weight of the rabbits averaged 1720 g. Urine collections were again made and the folic acid excretion determined. The results obtained for both experiments are shown in Table I.

Results and Discussion. The rabbits receiving the basal ration without added sulfa drug grew at a normal rate. The rate of gain for other groups which received 2.0 mg of pteroylglutamic acid per kg of ration was not increased. The rate of gain has been shown to increase when liver extract is added.[4] The addition of 2% succinylsulfathiazole to the ration markedly reduced the rate of gain of most rabbits, however the rabbits with a larger initial weight, particularly in the second experiment, were not severely retarded in weight gains. The rates of gain of the control groups included in the first experiment that received pteroylglutamic acid with or without the sulfa drug were normal.

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* We are indebted to Dr. L. D. Wright, Sharp and Dohme, Inc., for supplying the succinylsulfathiazole and to Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., for the synthetic pteroylglutamic acid used in this study.

† Present address: Division of Biochemistry and Nutrition, American Meat Institute Foundation, University of Chicago.

[1] Oleese, O., Pearson, P. B., and Schweigert, B. S., *J. Nutrition*, 1948, **35**, 577.

[2] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

[3] Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1945, **157**, 303.

[4] Kunkel, H. O., Simpson, R. E., Pearson, P. B., Oleese, O., and Schweigert, B. S., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 122.

TABLE I.
Effect of Ingesting Succinylsulfathiazole on Urinary Excretion of Folic Acid by the Rabbit.*
(μg excreted per rabbit per day).

Dietary regimen	Weeks on experiment 4	5	6	7	8	9	10
Exp. I							
Basal	2.3	1.5	2.6	3.6	10.9	4.8	8.9
Basal + S.S.	1.1	0.5	1.6	1.8	2.2	1.9	2.4
Exp. II							
Basal	2.6	3.0	7.8	10.0	—	3.8	3.4
Basal + S.S.	1.0	1.0	2.5	3.7	—	0.4	0.5

* The values are averages of 3 individual 3-day collections for the first experiment and of 6 individual collections for the second experiment.

The addition of 2% of succinylsulfathiazole markedly reduced the urinary excretion of folic acid active compounds. This effect was evident by the 4th week and although the excretion of folic acid remained at a low level for the succinylsulfathiazole group, a considerable increase in the apparent synthesis of folic acid was observed for the basal group, particularly from the 7th to 10th weeks. Although accurate measurements of folic acid in the ration were not achieved due to the low level, the estimated dietary intake of 1 μg per day approximated the amount excreted by the sulfa fed group and was much lower than the amount excreted by the basal group. It may be concluded therefore that the rabbit is capable of synthesizing sufficient folic acid, as well as pantothenic acid, riboflavin, and biotin, presumably by intestinal microorganisms to meet its needs and that the apparent synthesis of folic acid can be markedly reduced by the inclusion of 2% succinylsulfathiazole in the ration.

Other tests showed that the inclusion of succinylsulfathiazole in the ration did not reduce the hemoglobin level of the blood. Rabbit blood was found to contain folic acid conjugase;[5] however the level of apparent free folic acid in blood was very low (<1.0-4.0 millimicrograms per ml) and the data obtained do not permit an accurate evaluation of the folic acid blood levels of the groups receiving and not receiving the sulfa drug.

Summary. The urinary excretion of folic acid by rabbits fed purified diets with or without the addition of succinylsulfathiazole was studied. The inclusion of the drug markedly reduced the urinary excretion of folic acid. This effect was evident from the 4th to the 10th week of the experiments.

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[5] Simpson, R. E., and Schweigert, B. S., *Arch. Biochem.*, 1949, **20**, 32.

17012

Dissociation Among Lancefield's Group "B" Streptococci of Human and Bovine Origin.*

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One hundred and thirty bovine and 26 human strains of Lancefield's group "B" streptococci were studied within one month after isolation.

Primary isolations were made on beef heart infusion agar (Difco) which contained 3%

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* This investigation was conducted with the cooperation of the following institutions: San Juan City Hospital, Bayamón District Hospital and the Veterans Hospital at San Patricio.

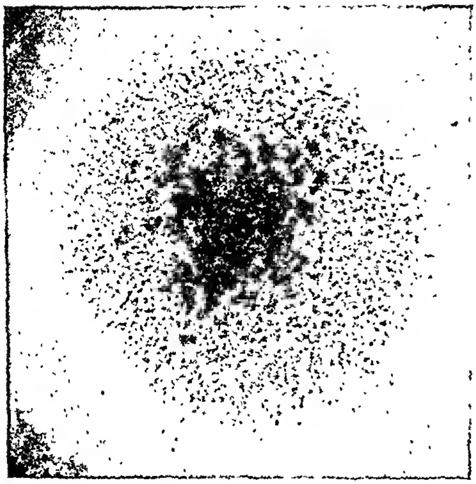

FIG. 1.
"M" colony, 60 hr, 37°C. Blood agar. × 49. Notice sectors indicating M ⟶ S variation. The granulations are most probably accumulations of red blood cells.

of rabbit or horse blood. The morphology of the colonies was studied on rabbit blood agar plates. The plates (streak) were incubated aerobically at 37°C and examined with a hand lens and a microscope. Only isolated colonies with ample free surrounding space were selected for study. In studying the colonial morphology by reflected light the plate had to be tilted at the proper angle in order not to miss certain details.

Four distinct morphological types were encountered among cultures recently isolated from milk or pus obtained from cows with clinical mastitis. A description of these follows:

1. Colonies slightly raised, comparatively large, about 2.5 mm after 48 to 72 hours incubation, edge entire or slightly wavy. Surface white and glossy by reflected light. With the low power of the microscope tiny white solid structures were seen scattered throughout the body of the colony giving to it the appearance of a tiny drop of slightly curdled milk. Consistency of thin paint. Semitransparent periphery and a rather *opaque central* area (Fig. 1). In the bacteria from some of these colonies definite capsules could be demonstrated either from young cultures on blood agar or in peritoneal exudate from mice. Broth cultures showed a homogeneous turbidity with a scanty powdery sediment. The cocci occurred singly, in pairs or in very short chains. Some strains were pathogenic and others were avirulent for mice. This description corresponds with the mucoid (M) colony of Dawson, Hobby and Olmstead.[1]

When colonies of this type were incubated at 37°C for 48 to 72 hours frequently wedge shaped, rather opaque outgrowths appeared at the periphery. (Fig. 1). These outgrowths when transplanted gave rise to rather opaque colonies which corresponded in all respects to the second type of colony encountered in primary cultures which we shall now describe.

2. Colonies raised, edge entire or very slightly wavy. Surface dull and usually slightly granular. Consistency of thick paint. They could easily be broken into *soft thick* membranous portions. Relatively opaque when compared with the "M" colonies. (Fig. 2). Growth in broth was homogeneous or very finely granular. The cocci occurred mainly in short chains. No capsules could be demonstrated. This description corresponds with the smooth (S) type colony of Dawson *et al.*[1]

It is apparent that the opaque wedge-like growths of the mucoid colonies represent a mucoid to smooth variation.

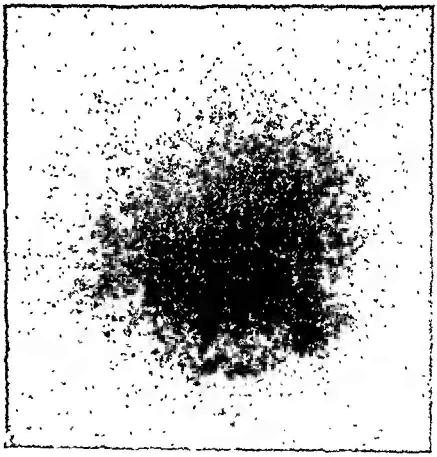

FIG. 2.
"S" colony. 48 hr at 37°C on blood agar. × 41. Granulations most probably accumulations of red blood cells.

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[1] Dawson, M. H., Hobb, G. L., and Olmstead, M., *J. Infect. Dis.*, 1938, 62, 138.

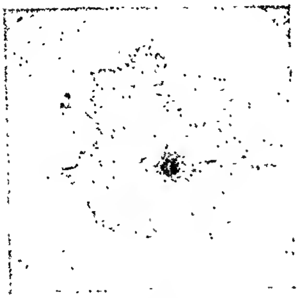

FIG. 3.
"M" colony with prominent rough outgrowths. One week on blood agar at 37°C. × 5.

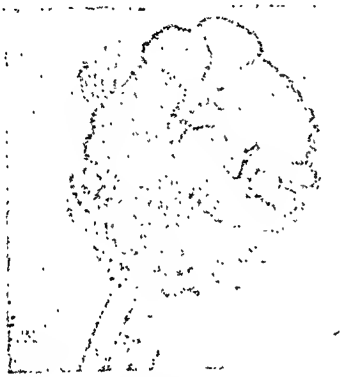

FIG. 4.
"S" colony with rough outgrowths. Four days on blood agar at 37°C. × 20.

If "S" or "M" cultures were left in the incubator from three days to one week rough, large, fan-like outgrowths usually arose from the periphery of the colonies. (Fig. 3 and 4). A large proportion of the "S" and "M" cultures produced these outgrowths. Transplants to broth from these outgrowths gave a cottony sediment with a clear supernatant as contrasted with the homogeneous turbidity obtained with "M" and "S" cultures. The cocci were larger than normal and occurred chiefly in long tangled chains. Colonies obtained from transplants of these rough sectors to 3% rabbit blood beef heart infusion agar showed a slightly raised conical central portion and a rather flat peripheral zone. Upon prolonged incubation they became flatter and flatter until their appearance was much like that exhibited by the "R" colonies obtained in primary cultures as we shall see later. This type of colony conforms in many respects with the "R" (rough) type of Dawson.[1]

3. Colonies prominently flat, composed of a thin membrane with the appearance and consistency of a spider web. Edge of colony very irregular and fimbriated. In some instances small, smooth papillae are distributed throughout the colony. (Fig. 5). Cottony sediment with clear supernatant in broth. Cocci usually larger than normal arranged in long

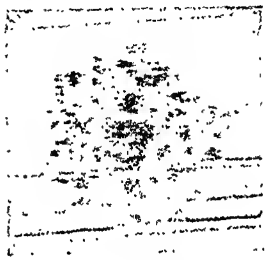

FIG. 5.
Papillated rough colony. × 15. Blood agar. Four days at 37°C.

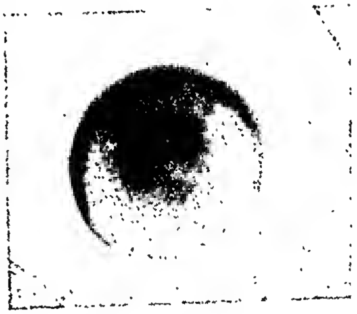

FIG. 6.
Twenty-four-hr-old precursor of smooth-ravined (SRa) colony. × 30.

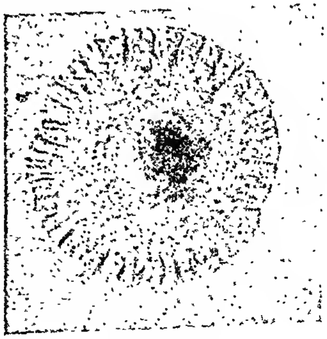

FIG. 7.
Smooth-ravined (SRa) colony. 72 hr on blood agar at 37°C. × 30.

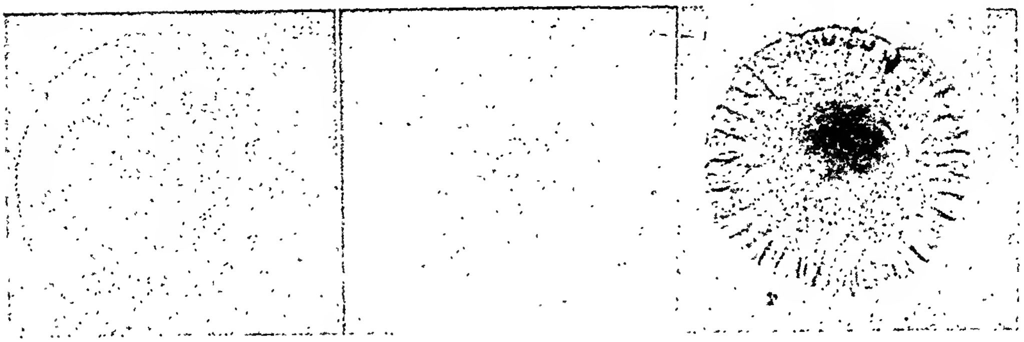

FIG. 8.
Stages in the development of the smooth-ravined (SRa) colony. Large type. Three-day-old colonies on rabbit blood agar. × 30.

tangled chains. These correspond with the rough (R) type of Dawson[1] and were very similar to the rough outgrowths already described obtained from "S" and "M" colonies.

4. Colonies were conical, with a prominently white, dense, raised central disc. The rest of the colony was much less dense. The whole colony was smooth and the surface glossy when 16 to 18 hr old and approximately 1 to 1:5 mm in diameter. (Fig. 6). A small and large type of colony were encountered. The central disc was usually of a soft consistency or exceptionally rather hard so that it could be removed with the wire without disturbing the rest of the colony which at this stage was of medium hardness and could be broken easily with an inoculating wire into thick membranous portions. Occasionally these colonies were of such consistency that they could be shifted from place to place without disturbing their characteristic morphology.

After from 48 to 72 hours incubation at 37°C the central white disc remained as described above. However, the rest of the colony showed by this time a deeply ravined glossy surface with a grayish milky appearance by reflected light. (Fig. 7). Now the colony was harder but it could be broken with the wire into thick membranous portions. The growth in broth was homogenous with scanty powdery sediment or finely granular with slightly foccular or slightly ropy sediment. The cocci occurred typically in chains of medium size. In some instances colonies in the different stages of development were encountered on the same plate (Fig. 8). So far as we know this type of colony has not been described before. We are designating it, and shall refer to it in this discussion as the "SRa" or the *smooth-ravined* colony.

If these smooth ravined cultures were left in the incubator at 37°C for 4 or 5 days some colonies showed glossy, soft pseudopod-like peripheral outgrowths. (Fig. 9). Upon prolonged continued incubation (one or two weeks) these smooth soft portions in turn gave rise occasionally to rough outgrowths similar to those already described as arising from "S" and "M" colonies.

All the morphological types described above were found in primary cultures from the mastitic udder. No rough (R) or *smooth-ravined*

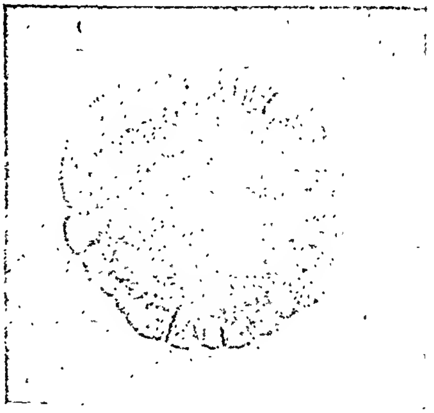

FIG. 9.
Smooth-ravined (SRa) colony with glossy outgrowth. Five days on blood agar. Large type of colony. × 30.

(SRa) types were found in primary cultures from human sources. All the human strains produced either "M" or "S" colonies. However, the S → R and M → R dissociations were produced with relative facility with human strains as described above for cultures of bovine origin.

The fermentative and other biological properties of most of these strains were tested[2] and no correlation was found between these properties and colonial morphology.

Serums prepared in rabbits with formalin treated suspensions of "M" cultures gave good precipitin reactions with formamide extracts of "M" or "SRa" organisms of human and bovine origin. Rabbit serum prepared with "S" or "SRa" suspensions reacted similarly. "M", "S" and "SRa" serums reacted negatively with formamide extracts prepared with the corresponding "R" variants.

The serums obtained from rabbits after several series of inoculations with formalin treated "R" organisms always gave negative precipitin reactions with formamide extracts of the homologous "R" suspensions or extracts prepared from the parent "M" or "S" human and bovine cultures.

These results show conclusively that the group precipitinogen extracted by Fuller's method is greatly diminished or completely lacking in the "R" phase of Lancefield's group "B" streptococci of human and bovine origin. One must keep in mind this fact in the identification of "R" variants of *Strep. agalactiae* obtained in primary cultures from the bovine udder.

Two "M" cultures which were virulent for mice (0.05 cc of 18 hr broth culture killed mice in 24 hours) gave rise to "R" variants which were completely avirulent (1 cc intraperitoneally).

All the bovine and human cultures, irrespective of their dissociative phase at the time they were tested, were resistant to streptomycin *in vitro* when tested by the filter paper disc method of Bondi *et al.*[3] All the bovine and human strains were sensitive to penicillin *in vitro* but the sensitivity varied greatly with the different strains. This variation in susceptibility to penicillin was independent of the origin and colonial morphology of the cultures.

Summary. The dissociation among 130 bovine and 26 human strains of Lancefield's group "B" streptococci has been studied. Four different morphological types of colonies (rough, smooth, mucoid and smooth-ravined) are discarded in detail. No correlation was found between colonial morphology and the biological properties and susceptibility to penicillin. All strains were resistant to streptomycin *in vitro*. Cultures from rough outgrowths of mouse virulent mucoid parent colonies were completely avirulent for mice. Formamide extracts of mucoid, smooth and smooth-ravined cultures reacted positively in precipitin tests with homologous and heterologous rabbit serums prepared with mucoid, smooth and smooth-ravined cultures and with commercial group "B" serum. Formamide extracts prepared from rough cultures always gave negative precipitin tests with good precipitating rabbit serums prepared with the corresponding mucoid, smooth and smooth-ravined parent cultures.

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[2] Pomales-Lebrón, A., Morales-Otero, P., and Baralt, J., PROC. SOC. EXP. BIOL. AND MED., 1947. **64**, 410.

[3] Bondi, A., Spaulding, E. H., Smith, D. E., and Dietz, C. C., *Am. J. Med. Sc.*, 1947, **213**, 221.

17013

A Histochemical Method for Localizing Cholinesterase Activity.*

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The exact site of cholinesterase (ChE) activity in relation to the structural elements of various tissues has been a matter of uncertainty. Information on this subject up to the present time has been derived indirectly from studies correlating the over-all ChE activities of different samples of tissues with the relative proportions of certain cells or structures present.[1,2] Gomori[3] has published a histochemical method for the direct visualization of the location of ChE activity, in which the substrates used are long-chain fatty acid esters of choline. However, his protocols indicate that the rate of splitting of these compounds by various tissues is extremely low in comparison with that of acetylcholine (ACh); with such enzyme sources as rat brain, which is said to contain only specific ChE,[4] and purified preparations of erythrocyte and electric organ ChE, little or no hydrolysis of these compounds occurred. It seems likely, therefore, that this technic localizes only nonspecific esterases, many of which hydrolyze ACh, but not specific ChE which is considered of physiological significance in the transmission of nerve impulses. In the method reported here, the substrate employed is acetylthiocholine (AThCh) which, as shown below, is hydrolyzed at a more rapid rate than ACh by both specific ChE and nonspecific esterases, presumably because of the weaker linkage of the $-\overset{\displaystyle O}{\overset{\|}{C}}-S-$ than of the $-\overset{\displaystyle O}{\overset{\|}{C}}-O-$ bond. The present technic appears to indicate the location of all enzymes classified under the general term "cholinesterase", and does not permit differentiation between the several types of ACh-splitting enzymes. Such a distinction is now being attempted by the use of the sulfur homologues of acetyl-beta-methylcholine and benzoylcholine as substrates, and by means of selective inhibitors.

Acetylthiocholine Iodide $(CH_3\overset{\displaystyle O}{\overset{\|}{C}}SCH_2CH_2\text{-}N(CH_3)_3I)$ was first synthesized by Renshaw *et al.*,[5] who noted that its pharmacological actions were similar to those of ACh but of briefer duration. We have employed their method of preparation with minor modifications.† Glick[6] reported that this ester was hydrolyzed more rapidly than ACh by horse serum ChE. The same relationship was found to hold for all sources of ChE tested in the present study, as shown in Table I. ChE activity was determined manometrically by a

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* This work was supported by the National Institute of Health, John and Mary Markle Foundation, and the Chalfant Fund.

[1] Marnay, A., and Nachmansohn, D., *J. Physiol.*, 1938, **92**, 37.

[2] Sawyer, C. H., and Hollinshead, W. H., *J. Neurophysiol.*, 1945, **8**, 137.

[3] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 354.

[4] Mendel, B., and Rudney, H., *Science*, 1943, **98**, 201.

[5] Renshaw, R. R., Dreisbach, P. F., Ziff, M., and Green, D., *J. Am. Chem. Soc.*, 1938, **60**, 1765.

† We are grateful to Dr. W. Gump of Givaudan-Delawanna, Inc., Dr. Max Tishler of Merck & Co., and Mr. P. W. Blume of Michigan Chemical Corp., for generous supplies of the initial compound, beta-chloro-ethyl dimethyl ammonium chloride. The latter organization supplies this substance commercially. Dr. Eric W. Martin, Philadelphia, Pa., suggested modifications of the synthesis. It has been brought to our attention that AThCh can be obtained from the Bios Laboratories, Inc., New York City.

[6] Glick, D., *J. Biol. Chem.*, 1939, **130**, 527.

TABLE I.

Rates of Hydrolysis of Choline Esters by Enzymes from Various Sources.

Enzyme source	Micromoles CO_2 per g or cc per hour		Ratio $\frac{\text{Q AThCh}}{\text{Q ACh}}$
	ACh	AThCh	
Rat Brain	224	367	1.64
" Erythrocytes	35	98	2.80
" Serum	23.4	62.3	2.67
Cat Erythrocytes	11.4	38.3	3.36
" Adrenal	50.6	104.5	2.06
Purified bovine Erythrocyte ChE (Winthrop-Stearns) 1.0 mg	1,820	2,700	1.49

modification of Ammon's[7] method. The initial concentration of each substrate was 5×10^{-3} M, which approaches the optimal range of ACh concentration for specific ChE,[8] and the pH of the solutions after gassing with 5% CO_2–95% N_2 was approximately 8.0. Parallel determinations with both substrates were run on all enzyme sources for periods of 10 to 30 minutes, depending on the rate of CO_2 production, and corrections were made for non-enzymatic hydrolysis. The concentration of Cu^{++} (0.002 M) employed in the histochemical procedure was found, under the same conditions, to produce no appreciable inhibition of rat brain homogenate ChE with either substrate, whereas 10^{-3} M di-isopropyl fluorophosphate (DFP) caused complete inhibition.

Histochemical Procedure. The method consists of incubating teased preparations or frozen tissue sections in a medium containing 4×10^{-3} M AThCh and 0.002 M copper glycinate at a pH of 8.06, saturated with copper thiocholine, for 10 to 60 minutes. The saturation of the substrate solution with the reaction product (in this case copper thiocholine) was first suggested in relation to enzymatic histochemical technics by Friedenwald and Becker.[9] It has the double advantage that diffusion of reaction product is greatly diminished or suppressed, thus enhancing the crispness of the histological localization, and, in addition, since the controls are incubated in the same solution and since the controls show no deposit, of demonstrating that the localization found in the test specimens is not due to mere staining or adsorption. Following this the sections are subjected to two brief rinsings in distilled water saturated with copper thiocholine, then immersed in ammonium sulfide solution, which converts the white precipitate of copper thiocholine to a dark brown amorphous deposit of copper sulfide. They are then affixed to slides with albumen, after which they are either mounted in glycerine or dehydrated through increasing concentrations of alcohol and counterstained as desired. Controls were run by allowing freshly-cut sections to stand in 10^{-3} M DFP in 0.85% saline for 30 minutes at room temperature, following which they were washed in distilled water and treated by exactly the same procedure as described above. In all preparations studied to date, the controls were completely free of the characteristic staining seen in the untreated preparations described below.

Reagents

Solution 1: 3.75 g Glycine, 18.0 cc N KOH, distilled water q.s. 100 cc.

Solution 2: 0.1 M copper sulfate.

Solution 3: 14.5 mg AThChI, 0.75 cc H_2O, 0.25 cc solution 2. Centrifuge. Decant supernatant solution from precipitated cupric iodide.

Copper Thiocholine prepared by alkalinizing a solution of AThCh in copper glycinate to pH 12.0 with KOH, allowing to stand overnight at room temperature, collecting the precipitate and washing with water.

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[7] Ammon, R., *Arch. ges. Physiol.*, 1933, **233**, 486.

[8] Augustinsson, K.-B., *Acta Physiol. Scandinav.*, 1948, **15**: Supplementum 15, 1.

[9] Friedenwald, J. S., and Becker, B., *J. Cell. Comp. Physiol.*, 1948, **31**, 303.

Incubation Solution. 0.4 cc solution 1, 0.2 cc solution 2, 8.6 cc H_2O; add small trace of copper thiocholine, disperse thoroughly, and place in water bath at 37° for at least 15 minutes, stirring occasionally. Immediately before using, add 0.8 cc solution 3, filter.

Results. The following descriptions and accompanying photomicrographs† are of preparations which were not counterstained. Other tissues studied to date, the interpretation of which has been less clear, include liver, spleen and heart.

Striated muscle. Teased preparations of intercostal muscle of the rat (Fig. 1, 2) following incubation for 10 minutes, revealed distinct structures identified as motor endplates. It is interesting to note that the picture bears a striking resemblance to that obtained of the same muscles of the mouse by Couteaux,[10] using the Janus green-ammonium

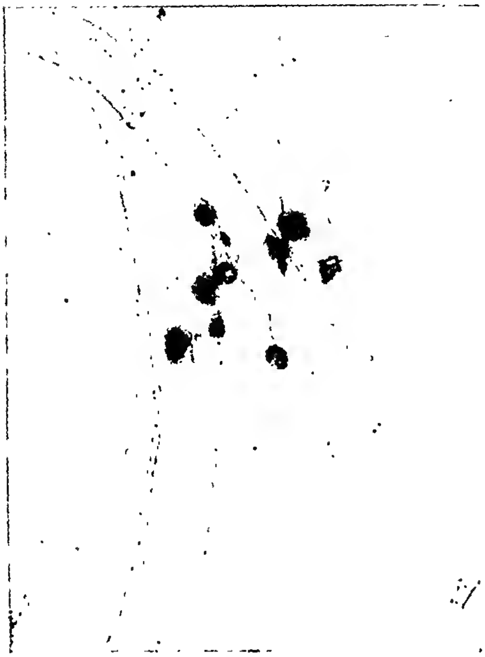

FIG. 1.
Rat intercostal muscle, × 125.

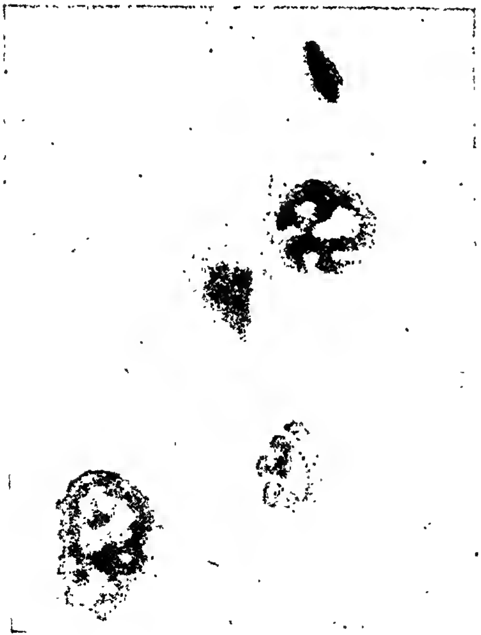

FIG. 2.
Rat intercostal muscle, × 600.

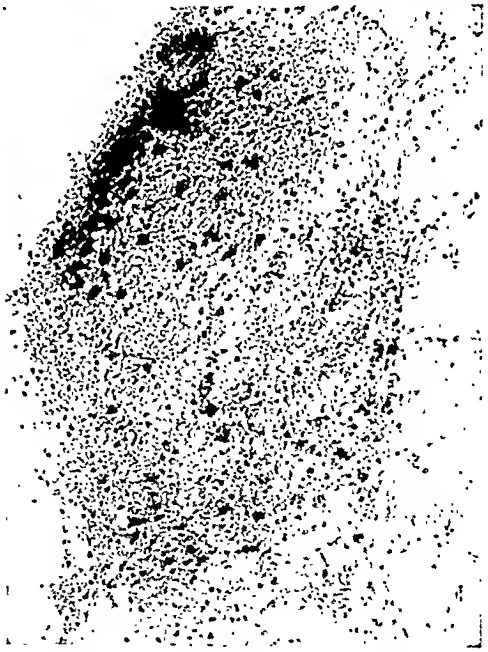

FIG. 3.
Rat medulla, region of gracile and cuneate nuclei, × 125.

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† Photomicrographs taken by Mr. Delbert Parker.

[10] Couteaux, R., *Rev. Canadienne de Biol.*, 1947, 6, 563.

molybdate stain which is considered selective for the subneural apparatus. Couteaux postulated that ChE is probably most concentrated in this region, a hypothesis with which the present findings are compatible. When preparations were allowed to incubate for longer periods, the sarcolemma and nuclei also showed varying degrees of staining.

Brain. Sections of rat medulla (Fig. 3,4), incubated for 40 minutes, were stained very deeply in the regions of the gracile and cuneate nuclei. At these sites large neurons and the immediate portions of their processes were revealed distinctly, as well as numerous glial nuclei which appeared to be predominately microglial. Because of the thickness (30 μ) of the sections studied with the present technic, it could not be determined whether or not the irregular background staining was due entirely to intracellular material. Elsewhere, cell bodies, processes and nuclei of fibrous astrocytes and nuclei of microglia were visible. The general architecture of the former appeared to outline various fiber tracts.

Autonomic Ganglia. Incubation of sections

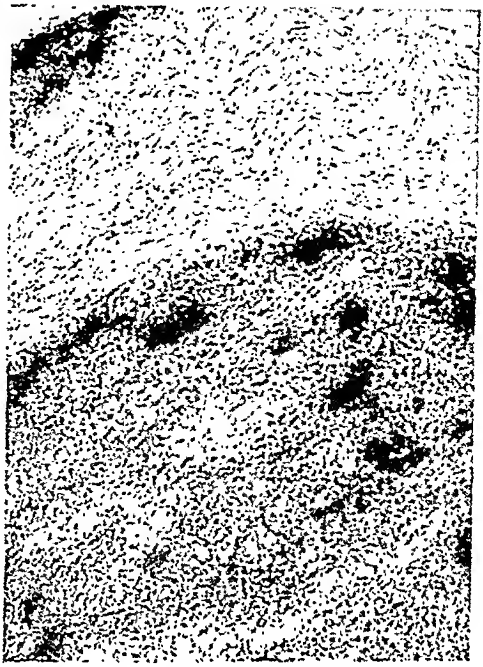

FIG. 5.
Cat superior cervical ganglion, × 125.

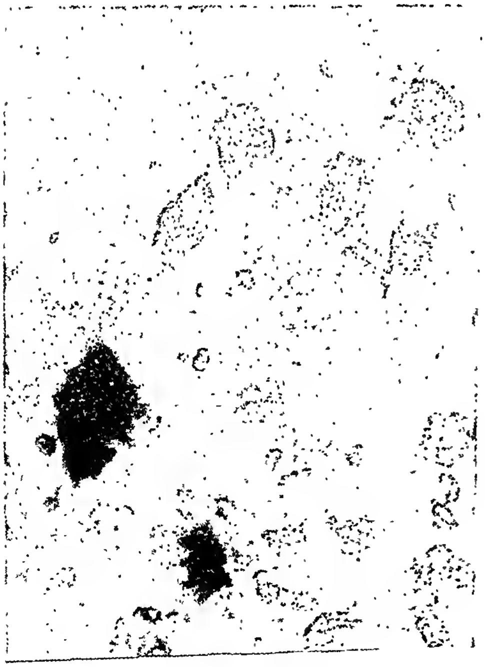

FIG. 4.
Rat medulla, region of gracile nucleus, × 600.

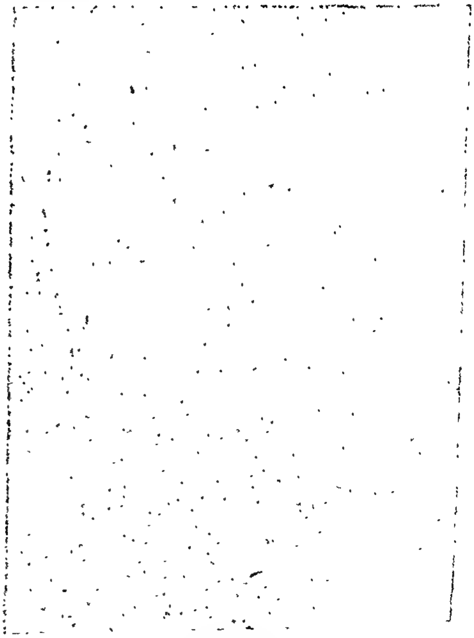

FIG. 6.
Cat superior cervical ganglion, previously incubated in 10^{-3} M DFP, × 125.

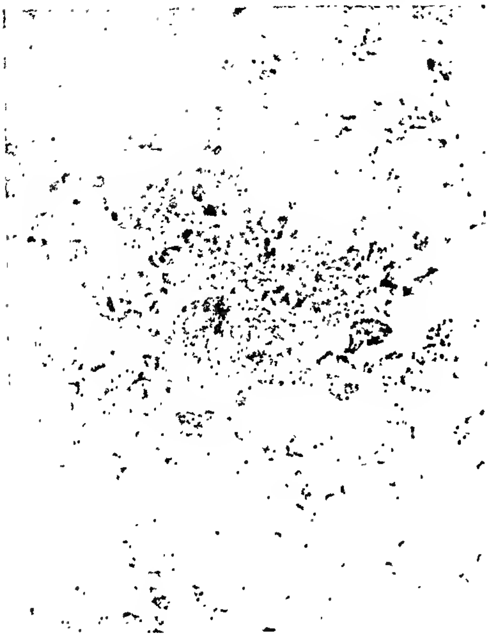

FIG. 7.
Cat superior cervical ganglion, × 600.

of cat superior cervical ganglion for one hour resulted in extremely dark staining (Fig. 5) which was completely lacking in the DFP-treated controls (Fig. 6). In the associated trunk, the nuclei of Schwann's cells were distinguishable, along with the general outline of the nerve fibers. Besides the heavily-staining ganglion cells and nuclei of satellite cells, both of which were seen better in sections incubated for one-half this period (Fig. 7), a fairly heavy background stain was present, forming an irregular pattern. No definite identification has been made of the structures represented by the discreet particles forming this background. It was much less dense in sections of the stellate ganglion of the same animal, where the ganglion cells, in consequence, stood out more sharply.

In sections of the rat ileum (Fig. 8), ganglion cells were stained in the areas of the submucous and myenteric plexi, and appeared to be far more numerous in the latter. The nuclei and general outlines of the muscle fibers were also stained.

Adrenal. The ganglion cells and nuclei of

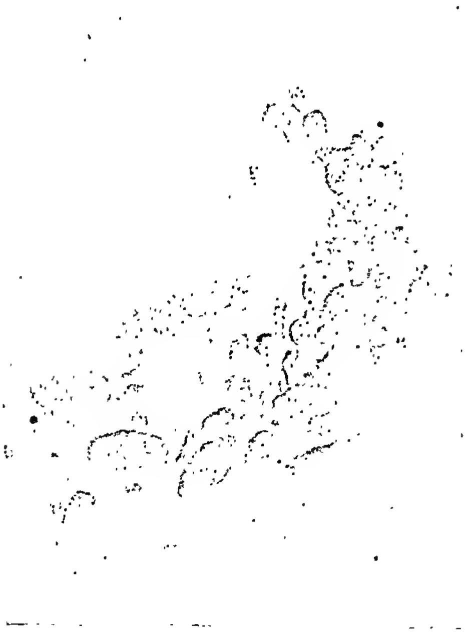

FIG. 8.
Rat ileum, region of myenteric plexus, × 600.

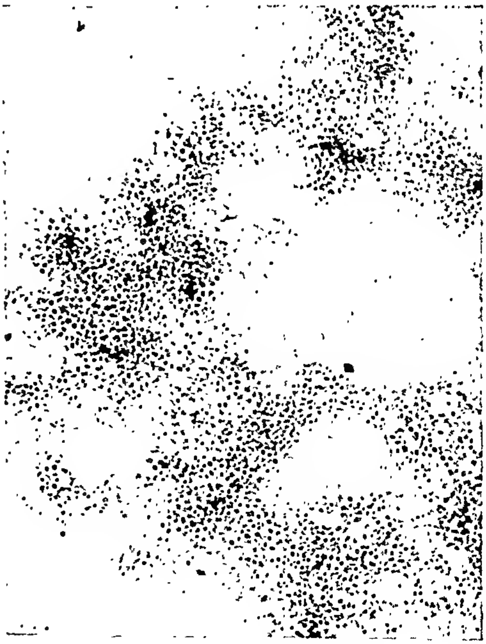

FIG. 9.
Cat adrenal medulla, × 125.

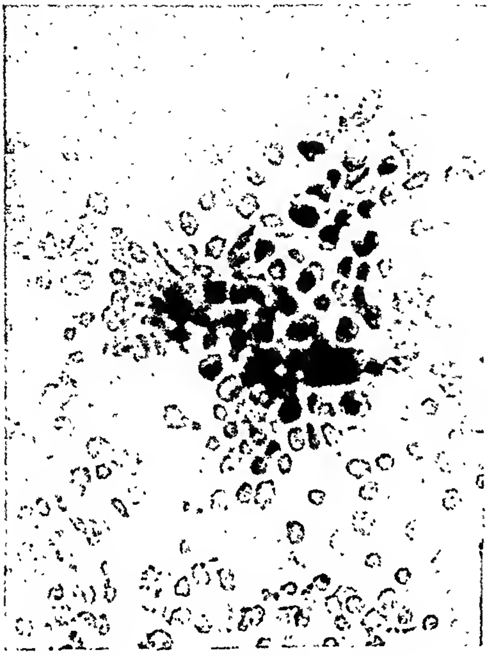

FIG. 10.
Cat adrenal medulla, × 600.

what appeared to be chromaffin cells of the adrenal medulla (Fig. 9, 10) of the cat were deeply stained, forming a pattern of irregular cords. No staining was seen in the cortex.

Comments. In any histochemical procedure the question arises as to whether or not the area where a precipitate is seen represents the actual site of enzymatic action. The alternative interpretation is that diffusion of the initial product of hydrolysis or of the subsequent reaction product has occurred, followed by a precipitation at an adjacent site. In the present study, the possibility of diffusion prior to precipitation was minimized by the previous saturation of the incubation medium with the final reaction product, copper thiocholine. The efficacy of this procedure in promoting immediate precipitation is of course dependent upon the adequate penetration of both copper thiocholine and copper glycinate from the surrounding medium to the sites of the enzyme in the tissues. That such occurred may be inferred from the fact that in preliminary tests in which saturation with copper thiocholine was omitted, precipitation occurred only after prolonged incubation (18-24 hours) and was manifested, after treatment with ammonium sulfide, by the appearance of clumps of acicular crystals of copper sulfide which bore no resemblance to cellular structures.

The intensity of the specific stain at any site is dependent upon several factors, foremost among which are probably the concentration of the enzyme and its reaction velocity or turnover number. The localization of specific ChE is favored in the present technic by a substrate concentration (4×10^{-3} M) in the optimal range for that enzyme; however, nonspecific esterases can also hydrolyze the substrate at this concentration, although at a slower rate. Methods which are being studied at present for localizing separately specific ChE and nonspecific esterases have been mentioned above.

Summary. A histochemical method is presented for localizing ChE activity by incubating tissue sections in a medium containing acetylthiocholine, copper glycinate and copper thiocholine. Results obtained with several tissues containing specific ChE are described and illustrated.

17014

Effect of Gold Administration on Liver Function in Dogs.

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It is a well-established fact that patients with rheumatoid arthritis experience much relief following the development of jaundice.[1] The jaundice apparently must be of the immediate direct reacting type (*i.e.* nonhemolytic), in order to be benficial. Hench[2] has suggested that the ameliorating effect of gold in rheumatoid arthritis is perhaps analogous to, if not basically identical with, that induced by intercurrent jaundice or pregnancy.

Hartung[3] found jaundice to be an uncommon sequela of gold salt therapy (2 in 800 cases). Altogether Hartung reports 4 patients who developed jaundice following a course of gold therapy. The onset of jaundice was accompanied by an improvement in the patients' subjective and objective symptoms, with relapses occurring almost immediately after the jaundice subsided. Hartung feels it to be highly questionable whether or not the jaundice was actually due to the gold salt therapy.

In this laboratory we have been concerned with the relationship of liver function to rheumatoid arthritis, and some of us[4] have described a method by means of which artificial jaundice of the direct reacting type may be produced. The present study was undertaken in order to ascertain the effect of gold administration on liver function.

Experimental. The following tests were carried out:

1. Rose Bengal Clearance[5]
2. Alkaline phosphatase[6]
3. Direct and indirect quantitative van den Bergh reaction for Bilirubin[7]

Two dogs, which were in good health and which weighed 13.2 and 14.1 kg were used.

Following the determination of control values for the above tests, 25 mg of gold in the form of sodium gold thiomalate (myochrysine) were administered to each dog intramuscularly each day for one week. Again their liver functions were tested. Next the gold administration was repeated, using 75 mg per day instead of 25. After one week liver function tests were repeated. Then, following a period of 2 weeks, the tests were again repeated.

Following this we administered 100 mg per day of gold thioglucose (solganal-B) in a 10% aqueous solution. Four days following the last administration, all the tests were repeated. The results are shown in Table I. It is apparent from the results that in no case did any of the liver functions tested, vary beyond the values accepted as normal.

Discussion. Rawls *et el.*[8] showed that patients with rheumatoid arthritis in many instances exhibit abnormal values for tests of liver function. Apparently considerable disturbance of liver function is needed before an amelioration of rheumatic symptoms is noted. Hence it appears unlikely that gold acts by producing sufficient effect on the liver to produce the relief from arthritic symptoms in this manner. On the other hand the poorly functioning liver of rheumatoid arthritis patients may be more easily open to the effect of gold therapy. In order to decide that, more quantitative studies on patients receiving gold therapy are needed.

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[1] Hench, P. S., *Med. Clin. No. Am.*, 1940, **24**, 1209.

[2] Hench, P. S., *Ann. Int. Med.*, p. 618, April, 1947.

[3] Hartung, E. F., *Med. Clin. No. Am.*, p. 553, May 1946.

[4] Snapp, F. E., Gutman, M., Li, T. W., and Ivy, A. C., *J. Lab. Clin. Med.*, 1947, **32**, 321.

[5] Stowe, W. P., Delprat, G. D., and Weeks, A., *Am. J. Clin. Path.*, 1933, **3**, 55.

[6] Shinowara, Jones and Reinhart, *J. Biol. Chem.*, 1942, **142**, 921.

[7] Hoffman, W. S., *Photelometric Clinical Chemistry*, New York, 1941, p. 231.

[8] Rawls, W. B., Weiss, S., and Collins, V. L., *Ann. Int. Med.*, 1939, **12**, 1455.

TABLE I.
Effect of Gold Therapy on Liver Function.

Dog	Alkaline phosphatase, units %	Rose Bengal clearance, %	van den Bergh reaction Direct, mg %	van den Bergh reaction Indirect, mg %
Control Values.				
1	6.2	119	0.0	0.2
2	4.4	109	0.0	0.1
Following 1 wk of daily administration of 25 mg of gold (sodium auro thiomalate).				
1	6.4	94	0.1	0.0
2	5.8	96	0.0	0.0
Following 1 wk of daily administration of 75 mg of gold (sodium auro thiomalate).				
1	7.2	111	0.1	0.0
2	8.2	111	0.1	0.0
Two wks later. No gold given during this interval.				
1	6.3	96	0.0	0.1
2	6.4	—	0.0	0.0
Following 1 wk of daily administration of 100 mg of gold thio glucose.				
1	7.0	115	0.0	0.0
2	6.4	95	0.0	0.0

In this study on dogs, the doses used, in terms of human therapy, were large, and a course of treatment which would require weeks in man is compressed into one week.

Summary. 1. Two dogs received gold in the form of sodium auro thiomalate and of thioglucose daily for a total of 3 weeks.

2. Determinations of Rose Bengal Clearance, alkaline phosphatase, and direct and indirect quantitative van den Bergh revealed no pathological changes in liver function.

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17015

Failure of Thyroidectomy and Thiouracil to Protect Rat Liver from Acute Carbon Tetrachloride Injury.

L. L. ASHBURN, W. H. BAKER, AND R. R. FAULKNER.
(Introduced by K. M. Endicott.)

From the Laboratory of Pathology and Pharmacology, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Md.

Leach and Forbes[1] have reported that sulfanilamide administered to rats protected the liver from inhaled carbon tetrachloride. In a subsequent experiment Forbes, Leach and Williams[2] found that sulfanilamide also retarded the development of hepatic cirrhosis induced by CCl_4. They suggested that the protective action might be related to the inhibition of thyroid activity known to be induced by sulfanilamide. György and Goldblatt[3] and György, Rose and Goldblatt[4] have shown that thiouracil and propylthiouracil exert a marked preventive effect on the incidence and degree of dietary cirrhosis in rats. These authors also suggested that the pre-

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[1] Leach, B. E., and Forbes, J. C., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 361.

[2] Forbes, J. C., Leach, B. E., and Williams, G. Z., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 47.

[3] György, P., and Goldblatt, H., *Science*, 1945, **102**, 451.

[4] György, P., Rose, C. S., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 67.

TABLE I.
Comparison of Liver Damage Produced by Carbon Tetrachloride in Thyroidectomized and Thiouracil-fed Rats and in the Pair-fed *and ad lib.* Controls.

Rat group	No. of rats	Extent of lesion*	Hydropic degeneration†	Necrosis‡	Fatty metamorphosis*
Thyroidectomized	13	++	+++	±	+
Pair-fed controls	13	++	+++	+	++
Ad lib. controls	11	±	+	∓	+
Thiouracil-fed	8	++	++	+	+
Pair-fed controls	8	++	++	+	+

* Extent of lesion, fatty metamorphosis—graded on a + to ++++ basis.

† Hydropic degeneration—plus values represent proportion of cells in involved areas showing this type of alteration.

‡ Necrosis —, ∓ rare cell, ± occasional cell, + a few cells.

ventive effect was mediated through the thyroid gland. Handler and Follis[5] in experiments with rats concluded "A decreased level of thyroid activity induced by thyroidectomy, thiouracil or p-aminobenzoic acid feeding prevents or retards the development of hepatic necrosis or fibrosis associated with choline and cystine deficiencies."

The experiments reported here show the failure of reduced thyroid activity in the rat to protect the liver against subcutaneously administered carbon tetrachloride.

Materials and Methods. The animals used were male rats of the Sprague-Dawley strain and weighed, at the beginning of the experiments, between 84 and 120 g. In one experiment 13 rats were thyroidectomized. An equal number of pair-fed rats served as one control group. A second control group of 11 rats were allowed diet *ad libitum*. The diet consisted of ground pellets (Purina laboratory chow) in which calcium carbonate was incorporated at a 2% level. The thyroidectomized rats were started on this diet 7 days before operation, and after operation were given 0.25 cc of 10% calcium gluconate intramuscularly for a few days, not exceeding 5 days for any rat. After 44 days all rats were injected subcutaneously with 0.05 cc CCl_4 (in equal parts of mineral oil) per 100 g of body weight, and killed and autopsied 24 hours later. The "thyroid area" in the operated rats was examined under 4X magnification for the presence of thyroid tissue; none was found.

In another experiment conducted at the same time, 8 rats were fed the ground pellet diet (without calcium carbonate) in which thiouracil was incorporated at a 0.1% level for 38 days and at a 0.2% level for another 45 days. The source of fluid for these rats was a saturated aqueous solution of thiouracil. A group of 8 pair-fed control rats received the same diet without thiouracil; water without thiouracil was the source of fluid. At the end of the 83 day period, all rats were injected with CCl_4 and killed as in the first experiment.

In each experiment the liver and thyroid gland or "thyroid area" of all rats were removed at autopsy and fixed in 10% formalin and prepared for microscopic study by paraffin embedding, and staining with azure eosinate. Frozen sections of the fixed livers were stained with oil red O.

Results. On microscopic examination the extent and type of liver cell damage were graded on a one plus to four plus basis. The liver cell injury, which was characteristically centrolobular in location, comprised loss of diffuse and granular cytoplasmic basophilia, irregular and inconstant slight cytoplasmic hyalinization, cytoplasmic oxyphilia, necrosis, hydropic degeneration and fatty metamorphosis. Of these changes only the last 3 listed, together with the extent of the lesion, were used as a basis of comparison. The results are shown in the accompanying table.

It is evident that the extent of the lesions and the degree of hydropic degeneration were not suppressed by thyroidectomy or thiouracil feeding. It is felt that the slight difference in amount of necrosis between the thyroidec-

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[5] Handler, P., and Follis, R. H., Jr., *J. Nutrition*, 1948, 35, 669.

TABLE I.
Effect of Gold Therapy on Liver Function.

Dog	Alkaline phosphatase, units %	Rose Bengal clearance, %	van den Bergh reaction	
			Direct, mg %	Indirect, mg %
Control Values.				
1	6.2	119	0.0	0.2
2	4.4	109	0.0	0.1
Following 1 wk of daily administration of 25 mg of gold (sodium auro thiomalate).				
1	6.4	94	0.1	0.0
2	5.8	96	0.0	0.0
Following 1 wk of daily administration of 75 mg of gold (sodium auro thiomalate).				
1	7.2	111	0.1	0.0
2	8.2	111	0.1	0.0
Two wks later. No gold given during this interval.				
1	6.3	96	0.0	0.1
2	6.4	—	0.0	0.0
Following 1 wk of daily administration of 100 mg of gold thio glucose.				
1	7.0	115	0.0	0.0
2	6.4	95	0.0	0.0

In this study on dogs, the doses used, in terms of human therapy, were large, and a course of treatment which would require weeks in man is compressed into one week.

Summary. 1. Two dogs received gold in the form of sodium auro thiomalate and of thioglucose daily for a total of 3 weeks.

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[1] Leach, B. E., and Forbes, J. C., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 361.

[2] Forbes, J. C., Leach, B. E., and Williams, G. Z., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 47.

[3] György, P., and Goldblatt, H., *Science*, 1945, **102**, 451.

[4] György, P., Rose, C. S., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 67.

Some of the tubes were heated for 5 minutes in a carefully controlled water bath at temperatures from 24° to 90°C. Other tubes were heated for 20 minutes at like temperatures. An electric photometer, with a 610 mμ filter, was adjusted to register 100% light transmission on the galvanometer scale, when the control tube was in place. (The light source, filter, absorption tube, and photocell were linearly arranged.) This value was recorded as incident light (I_0). Before each test the needle setting was adjusted with the control tube in place. The control tube was replaced by the tube containing the heated serum (which was cooled to room temperature) and the value on the galvanometer scale was recorded as transmitted light (I). The temperature of heating for each tube was increased after each photometric test until coagulation of the cell free fluid took place.

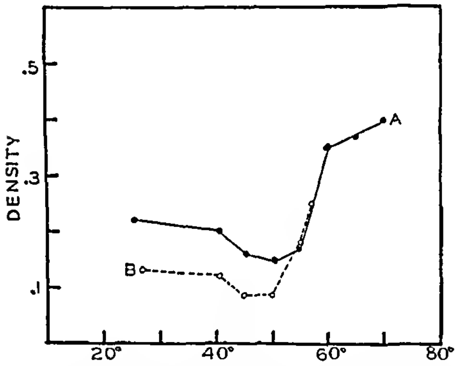

FIG. 1.

Graph obtained by plotting the optical density ($d = \log I_0/I$) against the temperature, in degrees Centigrade, at which the body fluid was heated. Curve A, fluid heated for 5 minutes; curve B, for 20 minutes.

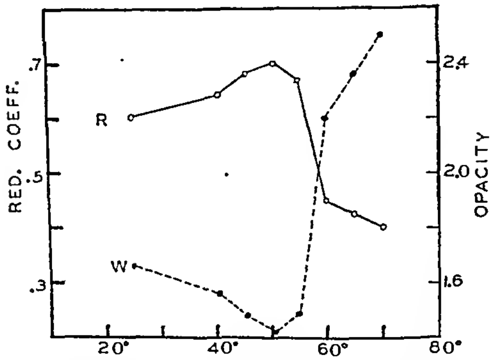

FIG. 2.

Graph showing the curves obtained by plotting the reduction coefficient ($r = I/I_0$) and the opacity ($W = 1/r = I_0/I$) against the heating temperature in degrees Centigrade. Curve R, reduction coefficient; curve W, opacity.

Results. The results are summarized in Fig. 1 and 2. Fig. 1 shows the results of plotting optical density ($d = \log I_0/I$) against temperature of heating. Curve A represents fluid heated for 5 minutes; curve B for 20 minutes. The results show that at about 40°C there is a decrease in optical density which suddenly increases rapidly beginning at about 50°C. (There is an evident increase in light transmission at about 40°C followed by a decrease beginning at 50° to 55°C).

Fig. 2 shows the results of plotting reduction coefficient ($r = I/I_0$) and opacity ($W = 1/r$) against temperature of heating. The curves are for fluid heated for 5 minutes. It is evident that at 40°C there begins an increase in reduction coefficient and a decrease in opacity. At about 50°C the reduction coefficient begins to fall, at first slowly, then rapidly and the opacity increases similarly. Curves for fluid heated for 20 minutes have the same configuration but are higher or lower on the graph.

The temperature for coagulation of the fluid varied greatly with different samples. This agrees with the results of Andrews who says that the fluid may coagulate at any temperature from 63° to 80°C.[2]

Discussion. The pooling of a series of experimental individuals to obtain directly a median value has been used for various investigations with favorable results.[3] In the tomized rats and their pair-fed controls is within the range of error inherent in the system of grading. Thyroidectomized rats showed less fatty metamorphosis; the difference is not great but may be significant. The livers of rats fed *ad libitum* showed extremely little damage except the one plus fatty metamorphosis. The only known differences between these rats and the pair-fed controls of this experiment were the greater amount of food consumed and a faster growth rate.

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[2] Andrews, E. A., *Johns Hopkins Univ. Circ.*, 1890, **9**, 65.

[3] Greiff, D., and Pinkerton, H., *J. Exp. Med.*, 1945, **82**, 193.

The few sections made through the "thyroid area" of each operated rat failed to reveal any thyroid tissue. The thyroid gland of all thiouracil fed rats showed marked epithelial hypertrophy with severe depletion of colloid. The thyroid glands of all other rats were histologically normal.

Discussion. The inhibition of thyroid activity suggested by Forbes *et al.*[2] as a possible explanation for the liver protective effect of sulfanilamide is not supported by our findings. It should be pointed out, however, that in their experiment the CCl$_4$ was administered by inhalation.

György *et al.*[3] and Handler *et al.*[5] found that a decreased level of thyroid activity protected the livers of rats against parenchymal necrosis in dietary cirrhosis studies. In view of the findings in the present study it would seem that the mechanism of protection against carbon tetrachloride necrosis[2] and the necrosis occurring in dietary cirrhosis is different. In this connection Handler *et al.*[5] found that sulfasuxidine lessened the tendency of the livers of choline-deficient rats to become necrotic and scarred even though the thyroid glands of these animals were essentially normal.

Summary. The lowering of thyroid activity by (1) thyroidectomy and (2) the feeding of thiouracil in a stock diet, failed to prevent or suppress hydropic degeneration or necrosis of the rat liver induced by subcutaneous administration of carbon tetrachloride.

17016

Effect of Temperature on Transmission of Light by Cell Free Body Fluid in *Phascolosoma gouldii.*

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The biological fluids of higher animals have been studied intensively and extensively from the point of view of chemical, physical, and physiological characteristics. The physical nature of the serum of mammals, for example, has been investigated with great thoroughness by Du Noüy who showed that at 56°C "profound modifications in the structure of the proteins and of the lipoprotidic complex" become evident.[1]

On the other hand, very little is known about the physical behavior of invertebrate body fluids. In order to ascertain the relationship of invertebrate fluids to those of higher animals, a series of experiments was made to test the effect of heating on the transmission of light by the cell free body fluid in *Phascolosoma gouldii.*

Material and methods. In general the procedure was similar to that followed by DuNoüy in studies on horse serum. Body fluid was carefully removed from 10 phascolosomas, using a 5 cc hypodermic syringe fitted with an 18 gauge needle. Approximately 1 to 3 cc of pink fluid were obtained from each worm. The pooled fluid was then centrifuged for about 10 minutes in an ordinary clinical cen-

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[1] Du Noüy, L., *Studies in biophysics. The critical temperature of serum (56°).* New York, 180 pp. 1945.

17017

Observations on the Racial Distribution of Variants of Blood Type rh′.

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Following the demonstration of the existence of 3 major varieties of Rh factors, determining 8 types of human blood, it was found that the incidence of the Rh types differed in different races.[1] It was also found that bloods existed which failed to give a clear-cut positive or negative reaction with one or more of the 3 reagents, anti-Rh_0, anti-rh′, or anti-rh″, indicating the existence of variants of each of the Rh factors.[2,3] In the earlier work these aberrant bloods were designated as belonging to "intermediate" types in order to indicate that the reactions obtained with the reagents were weak or intermediate in intensity. It was observed that the so-called "intermediate" types were particularly frequent among Negroids.[4] The purpose of the present communication is to present the results of a study of so-called "intermediate" types on a larger series of cases, and to discuss the general significance of the findings.

The donors who provided the blood for the present study were individuals who presented themselves at the Blood Bank of the New York University-Bellevue Medical Center of which one of us (L. J. U.) is the Director. They therefore represent a random group who presented themselves consecutively. The tests were carried out in the usual manner, using potent specific reagents of the tube agglutinating variety. For the sake of simplicity, the present paper will be limited only to the results of tests for the rare blood type rh′ and its variants.

In Table I is shown the relative incidence of blood type rh′ among Negroids and Caucasoids. Whereas only 2.7% of the donors who presented themselves belonged to the Negroid race, among those individuals belonging to type rh′ fully 13.9% were Negroids. This indicates that the type rh′ is about 4 to 5 times as frequent among Negroids as among Caucasoids.

As indicated above, some type rh′ bloods gave weak or indefinite reactions when tested with anti-rh′ serum. In Table II is shown the relative incidence of such weakly reacting or "intermediate" bloods among Negroids and among Caucasoids. It will be seen that while only 5% of type rh′ bloods among Caucasoids gave weak reactions with anti-rh′ serum, 27.6% among Negroids gave weak reactions. Therefore, these results on a larger series of cases confirm the previous report concerning the more frequent occurrence of bloods of "intermediate" types among Negroids.[4]

Comment. With regard to the significance of the so-called "intermediate" types, this is best explained by drawing an analogy with the varients of group A. When earlier workers observed that group A bloods fall into two natural subdivisions, namely, those giving strong reactions and those giving weak reactions, they explained these observations on a quantitative basis, believing that weakly reacting bloods contained less agglutinogen A than strongly reacting bloods. From the work of Landsteiner,[5,6] we know the difference in intensity of the reactions of group A bloods does not represent a quantitative difference but instead a qualitative difference. Antibodies and antigens behave in reactions like locks and keys. A key designed for lock X may also open lock Y, but usually not quite present work it was essential in order to have large enough samples for measurement. The median values are quite consistent.

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[1] Wiener, A. S., Sonn, E. B., and Belkin, R. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 238.

[2] Wiener, A. S., *Science*, 1944, **100**, 595.

[3] Wiener, A. S., Davidsohn, I., and Potter, E. L., *J. Exp. Med.*, 1945, **81**, 67.

[4] Wiener, A. S., Unger, L. J., and Sonn, E. B., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 89.

[5] Landsteiner, K., and Witt, D. H., *J. Immunol.*, 1926, **11**, 203.

[6] Landsteiner, K., and Levine, P., *J. Immunol.*, 1926, **12**, 441.

If it is assumed that the body fluid contains protein particles dispersed in a fluid medium, then if the particles become smaller the light transmitted shifts toward the red end of the spectrum.[4,5]

It is well known that electric photometers are ideal for measuring the light transmitted in a parallel direction through a turbid medium.[6] In the present work a red filter was used in such an instrument. Any shift, therefore, in light transmission toward the red end would be reflected in a decrease in density and opacity values of the fluid. Such a decrease is evident after heating the body fluid to 40° or 50°C. This increase in transmitted light may be interpreted as indicating a decrease in the size of the particles in solution. Continued heating above 50° C results in a pronounced increase in optical density indicating a similar increase in the particle size until finally coagulation takes place.

No measurements were made of light scattered in relation to light absorbed by the body fluid. Consequently, it is not certain whether scattering or absorption or a combination of both is responsible for the decrease in transmitted light. Measurements of scattered light are now in progress. However, in other investigations there has been found a consistent parallelism in serological tests between results obtained from measurements of scattered light and those of transmitted light.[7]

The present results are especially interesting if compared with those obtained by Du Noüy using horse serum. His figures indicate no change in light transmitted until the serum is heated to about 56°C at which point there is a pronounced decrease in transmitted light. The consistent preliminary increase in light transmitted after heating to 40° to 50°C, which is found in the body fluid of *Phascolosoma,* does not obtain in horse serum.

Apparently, heating causes the particles in the body fluid in *Phascolosoma* first to decrease in size, perhaps by giving up water, and then to increase in size either by hydration or by aggregation.

The results indicate what might be called 2 critical temperatures for the cell free fluid in *Phascolosoma*: a) 40°C above which heating brings about a decrease in particle size; b) 50°C above which particle size is increased possibly by "intramolecular hydration".[1]

The present method gives a new approach to the study of the phylogenetic relationships of animal body fluids. It is anticipated that a series of investigations covering representative invertebrates and vertebrates will be made with the view to ascertain the evolutionary development of the complex sera of higher animals.

Summary. Cell free body fluid from *Phascolosoma gouldii* was heated in sealed tubes at temperatures from 24° to 90°C. Measurements of light transmitted by the heated fluid indicate that there are 2 critical temperatures for the fluid: a) 40°C above which heating brings about a decrease in particle size; b) 50°C above which particle size is increased.

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[4] Burns, D., *An introduction to biophysics,* Macmillan, New York. 580 pp. 1929.

[5] Ostwald, W., and Fischer, M. H., *An introduction to theoretical and applied colloid chemistry.* New York, 266 pp. 1922.

[6] Drabkin, D. L., Photometry and spectrophotometry, in *Medical Physics,* Chicago, 1744 pp., 1944.

[7] Baier, J. G., *Physiol. Zool.,* 1947, **20**, 172.

17018 P

Veratramine, an Antagonist to the Cardioaccelerator Action of Epinephrine.

Otto Krayer.

From the Department of Pharmacology, Harvard Medical School, Boston, Mass.

Hitherto studied adrenolytic substances prevent or abolish the vasomotor effect of epinephrine but are unable to modify its effect upon heart rate. Consistent results, obtained in 10 heart-lung preparations of the dog and in 4 spinal cats, show that veratramine,*[1,2] one of the veratrum alkaloids recently reviewed,[3] prevents or abolishes the cardioaccelerator action of epinephrine in doses which do not abolish its vasopressor effect.

In the heart-lung preparation with a total blood volume of 500 to 900 cc[4] the heart rate can be brought to a steady level of between 200 to 260 beats per minute by the continuous infusion of 0.33 to 0.65 cc of epinephrine 1:100,000 per minute. One milligram of veratramine promptly reduces the rate to or near the normal level without modifying the regular sinus rhythm. (Fig. 1).

The veratramine effect can be overcome, at least partially, by sufficiently high concentrations of epinephrine. Atropine in doses up to 10 mg increases heart rate by 10 to 20%, as in the normal heart-lung preparation, and does not abolish the effect. The veratramine action is long lasting. After one milligram it does not begin to wear off within one hour in the heart-lung preparation. In the pithed cat, however, the effect disappears faster.

After pretreatment with veratramine, ex-

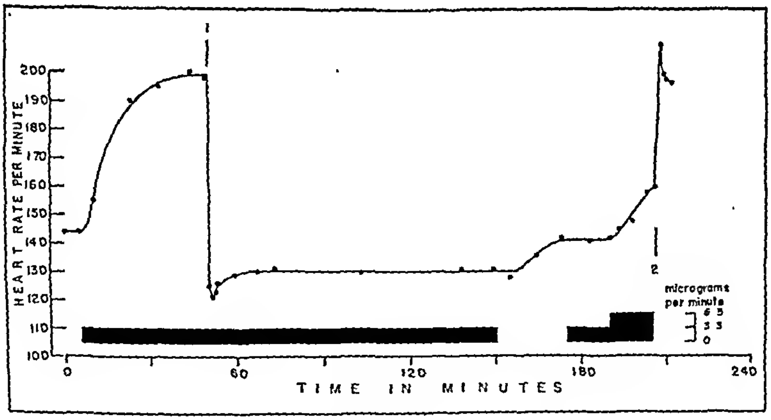

Fig. 1.

Effect of veratramine on cardioacceleration by epinephrine. Heart-lung preparation. Dog, male, 11.6 kg. Total blood volume 800 cc.

Black bar: Continuous infusion of epinephrine tartrate; calibration on right in micrograms of base.

Signal 1: Injection of 1 mg veratramine.

Signal 2: Injection of 500 μg epinephrine (as tartrate) in 5 seconds close to right atrium.

Temperature of blood between 38.6 and 37.6°C.

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* Generously supplied by Prof. W. A. Jacobs of the Rockefeller Institute, New York.

[1] Saito, K., *Bull. Chem. Soc. Japan*, 1940, **15**, 22.

[2] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, 1945, **160**, 555.

[3] Krayer, O., and Acheson, G. A., *Physiol. Rev.*, 1946, **26**, 383.

[4] For details of method see Krayer, O., and Mendez, R., *J. Pharmacol. and Exp. Therap.*, 1942, **74**, 350.

TABLE I.
Relative Incidence of the Blood Type rh′ Among Negroids and Caucasoids in New York City.

Types of donors	No. of consecutive donors	Caucasoid (%)	Negroid (%)
All Rh types	98,529	97.3	2.7
Type rh′ only	705	86.1	13.9

TABLE II.
Relative Incidence of Weak and Strong Reactions Among Type rh′ Bloods.

Race	No. consecutive type rh′ donors	Strong reactions (%)	Weak reactions (%)
Negroid	98	72.4	27.6
Caucasoid	607	95.0	5.0

as smoothly as in the lock for which it was designed. On the other hand, keys could be designed for lock Y which may or may not open lock X. Similarly, the common anti-A agglutinin clumps A_1 cells somewhat more strongly than A_2 cells, presumably because the configuration of the antibody molecule more closely conforms with A_1 than with A_2. On the other hand, A_2 blood contains other structures which are lacking or different from those present in A_1 blood, as shown by its reactions with anti-O serum. In the same way the existence of type rh′ bloods giving weak or "intermediate" reactions does not necessarily mean that the quantity of rh′ factor in such bloods is smaller than in typical type rh′ bloods, but rather that the rh′ factor is qualitatively different. In support of this interpretation may be cited the observation that among Caucasoids some type Rh_1 bloods which react weakly with anti-rh′ serum are strongly clumped by anti-rh^w (C^w) serum.[7] It may be of interest to mention that despite the relatively high incidence of weak rh′ bloods among Negroids, thus far we have failed to demonstrate the presence of rh^w factor in any blood of Negroid origin in tests on a random series of 105 individuals. No doubt with more intensive and prolonged study, antiserums will be found or produced which strongly clump other "intermediate" rh′ bloods such as described here, while giving weak or no reactions with "typical" rh′ bloods. This same principle would also apply to bloods giving "intermediate" reactions with anti-Rh_0, anti-rh″, anti-hr′, and anti-hr″ sera.

It must be emphasized that one is not justified in giving a specific name to an "intermediate" blood except when a specific antiserum has been found, as in the case of Rh_1^w bloods which contain the special factor rh^w. In the present stage of our knowledge concerning the "intermediate" Rh_0 factors, the name D^u is misleading and possibly incorrect, so long as no specific factor or factors corresponding to D^u have been isolated.[8]

Summary. Observations on a large series of blood donors indicate that the rare type rh′ is about 4 to 5 times as frequent among Negroids as among Caucasoids. Moreover, it is found that type rh′ bloods giving weak or "intermediate" reactions are about 5 to 6 times as common among Negroids as among Caucasoids, confirming previous observations on a shorter series of cases. It is pointed out that the existence of bloods giving weak reactions with anti-rh′ serum is more reasonably explained by postulating the existence of qualitative variants of the rh′ factor such as rh'^w, than by attributing the weak reactions to the presence of a smaller quantity of the factor rh′ in the blood. A similar explanation would apply to certain bloods giving "intermediate" reactions with sera anti-Rh_0, anti-rh″, anti-hr′, and anti-hr″.

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[7] Wiener, A. S., and Gordon, E. B., in preparation.

[8] Race, R. R., Sanger, R., and Lawler, S. D., *Annals Eug.*, 1948, **14**, 171.

17019

Effect of 1-(3,4, dihydroxyphenyl)-2-Isopropylaminoethanol, (Isopropyl-epinephrine) on the Rhythmic Property of the Human Heart.

M. H. NATHANSON AND H. MILLER.*

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In previous studies[1] a method was described and utilized for the study of the action of drugs on the property of rhythmicity or automaticity of the human heart. The method depends on the fact that it is possible in many individuals especially elderly males, to produce consistently a cardiac standstill of many seconds duration by compression of the carotid sinus. The prolonged cardiac arrest is the result of 2 factors (a) a temporary suppression of the sinus node depriving the heart of its normal pacemaker, and (b) the failure of development of secondary foci of impulse initiation. Following the administration of a variety of unrelated drugs including digitalis, caffeine, coramine, metrazol, barium chloride, calcium gluconate and thyroxin, the cardiac standstill could be consistently reproduced indicating that these compounds were ineffective in stimulating the pacemaking or rhythmic function of the heart. It was found that epinephrine consistently abolished the standstill either by stimulating the normal pacemaker or by initiating new rhythmic foci. This action was found in a number of epinephrine-like compounds (sympathomimetic amines) which were studied. All of the amines which were tested possessed a pressor action. However, it was noted that the increase in cardiac rhythmicity persisted after the blood pressure had returned to normal.

Recently a new sympathomimetic compound has been introduced as a substitute for epinephrine in the treatment of asthma. This compound is the N-isopropyl homologue of epinephrine, 1-(3,4, dihydroxyphenyl) 2-isopropylamino-ethanol. The pharmacological properties of this compound have been described by Lands and his associates.[2] The striking difference in the action of this compound, as compared with epinephrine, is that it does not exert a pressor action, showing usually a moderate depressor effect, especially a lowering of the diastolic pressure.

The study of the effects of a non-pressor compound on the rhythmic property of the heart seemed to be of both practical and theoretical interest. From a practical standpoint, many patients who have heart block or a sensitive carotid sinus, conditions in which a cardiac arrest may occur, have an associated hypertension. The use of a non-pressor compound would be of practical value in such conditions. From a theoretical standpoint, it seemed possible that the mechanism of the increase in cardiac rhythmicity by epinephrine might be clarified. Allen[3] reported that the cardiac effects of epinephrine are largely secondary to the pressor action of the drug. Moe and his associates[4] also concluded that the rise in blood pressure greatly facilitated the induction of rhythmic foci by epinephrine. However, Garb and Chenoweth[5] recently reported observations indicating that a sudden rise in arterial pressure was not necessary

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* Research Assistant, University of Southern California, Department of Cardiology, directed by Dr. George C. Griffith.

This study was carried out with the aid of the Dorothy H. and Lewis Rosenstiel Foundation.

[1] Nathanson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1933, 30, 967; *Arch. Int. Med.*, 1933, 51, 387; *Arch. Int. Med.*, 1934, 54, 111.

[2] Lands, A. M., Nash, V. L., McCarthy, H. M., Granger, H. R., and Dertinger, B. L., *J. Pharm. and Exp. Therap.*, 1947, 90, 110.

[3] Allen, W. F., *J. Pharm. and Exp. Therap.*, 1934, 50, 70.

[4] Moe, G. K., Malton, S. D., Freyburger, W., and Rennick, B., *Proc. Cent. Soc. Clin. Research*, 1947, 20, 24.

[5] Garb, S., and Chenoweth, M. B., *Fed. Proc.*, 1948, 7, 220.

TABLE I.

Positive Inotropic Without Positive Chronotropic Action of Epinephrine on Heart in Spontaneous Failure Pretreated with Veratramine. Heart-lung preparation. Dog, male, 9.8 kg. Total blood volume 500 cc. Blood temperature between 39 and 38.5°C.

Time	Heart rate per min.	Systemic output[*] in cc		Mean pressure			Remarks
		per min.	per stroke	Arterial mm Hg	Pulm. mm water	Right atrial mm water	
1:27 P.M.	163	420	2.6	80	158	27	
1:29	162	420	2.6	80	158	28	Between 1:38 and 3:45 8,5 mg veratramine adm. in divided doses
3:45	105	220	2.1	68	240	76	3:45 heart rate regular, normal sinus rhythm
3:47	105	205	2.0	67	242	77	3:47′ 55-60″ 10 μg epinephrine inj. in 5 sec. (as tartrate)
3:48	105	450	4.3	78	185	19	
3:49	95	420	4.4	78	185	21	
3:51	93	390	4.2	77	198	27	
3:54	96	360	3.8	75	206	34	
3:57	98	330	3.4	72	225	44	
3:58 0- 5″							3:58′ 0-5″ 30 μg epinephrine inj.
10-20″	110			84			
20-30″	127			78	173	10	
30-60″	97			76	185	15	
3:58 total	105	530	5.2[†]				
3:59	95	440	4.6	76	175	11	
4:01	95	430	4.5	76	180	13	

[*] Systemic output = total output of left ventricle minus coronary flow.

[†] Average stroke volume.

perimental heart failure can be relieved by epinephrine without cardioacceleration. (Table I.). In the normal heart-lung preparation 10 and 30 μg (as used in the experiment of Table I) lead to a maximal heart rate increase of at least 20% and 40% respectively; 10 to 15 and 20 to 30 minutes respectively are required for the initial rate to return. It is thus possible to separate the positive chronotropic from the positive inotropic effect of epinephrine. The rate of disappearance of epinephrine appears unchanged, as the positive inotropic action lasts as long as without the presence of veratramine.

Of other veratrum alkaloids studied jervine in a dose of one mg gave an effect similar to 0.1 mg of veratramine, while cevine in a dose of 40 mg was ineffective under the same conditions.

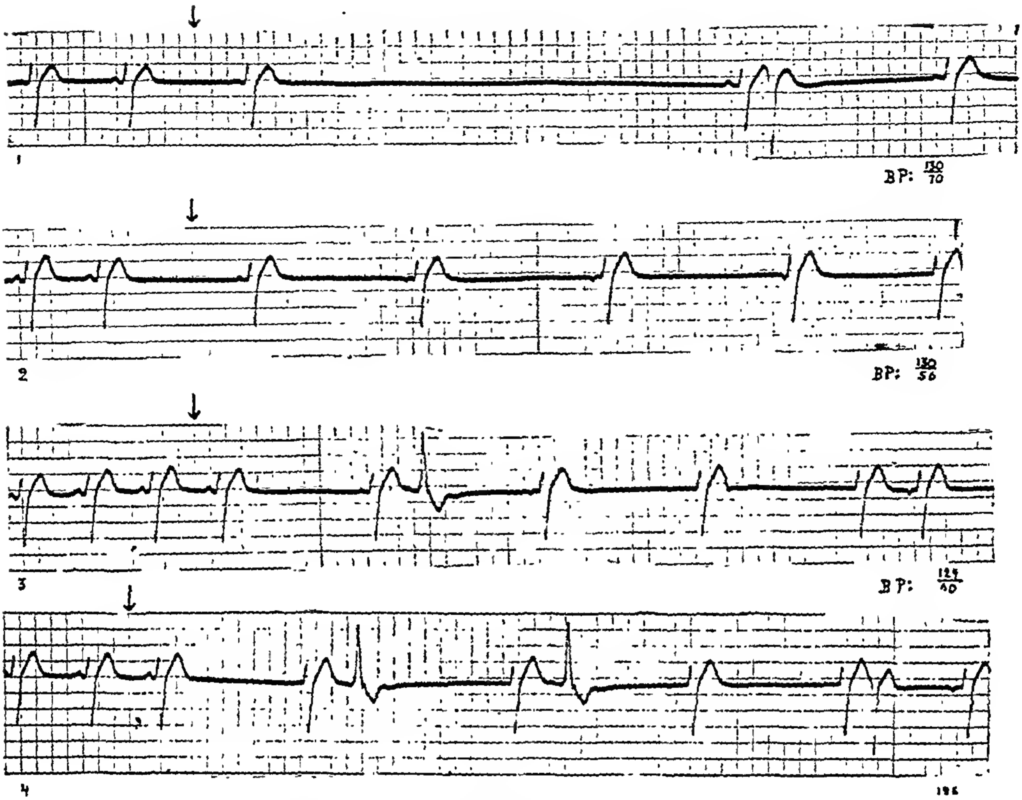

FIG. 2.

Pt. D.C. Strip 1 shows a cardiac standstill of 6.4 seconds duration induced by pressure on the right carotid sinus (arrow). Strips 2, 3 and 4, taken 7, 10 and 15 minutes after a subcutaneous injection of 0.15 mg of isopropylepinephrine. Carotid sinus pressure (arrow) fails to produce a standstill due to the development of beats from nodal and lower auricular foci and of occasional beats from a lower ventricular center.

in four and slightly depressed in 4 experiments. The diastolic pressure was depressed in every instance, in 6 experiments the effect was a reduction of 20 mm or more. There was a widening of the pulse pressure in every case.

Another method for the study of a sympathomimetic action on the heart is the application of the drug to patients with complete heart block. An increase in ventricular rate is an indication of the effectiveness of a sympathomimetic drug on the rhythmic function of the ventricular pacemaker. After a control electrocardiogram was made, isopropylepinephrine 0.2 mg was administered subcutaneously to 5 patients with complete heart block. Electrocardiograms were made at 5 minute intervals for 15 minutes and then at 15 minute intervals. The observations were carried out for 2 hours in 2 instances and one hour in 3 experiments. There was a definite increase in the ventricular rate in every instance. Table I.

Discussion. Isopropylepinephrine, a nonpressor compound, has a potent sympathomimetic action on the heart as indicated by the abolition of a carotid sinus induced cardiac standstill, by the modification of the ventricular complex of the electrocardiogram and by an increase in the ventricular rate in complete heart block. These observations indicate that a pressor action is not essential for the production of an epinephrine-like effect on the heart. The absence of a pressor action may be of practical value in the treatment of heart block or carotid sinus asystole associated with hypertension.

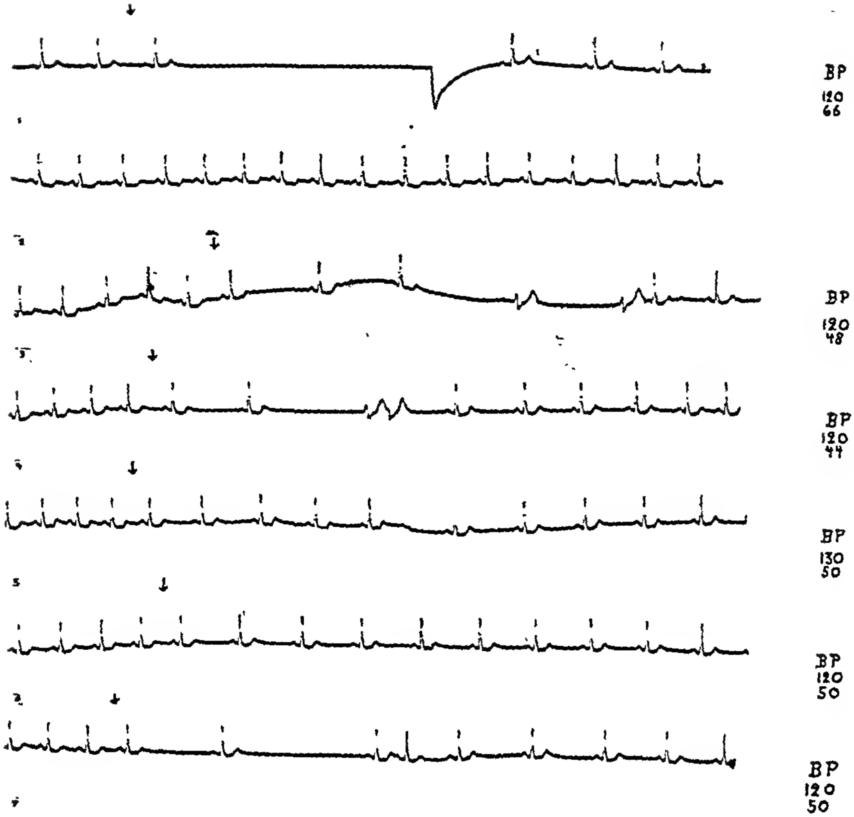

FIG. 1.

Pt. P.L. Strip 1, shows a cardiac standstill of 6 seconds duration induced by pressure on the right carotid sinus (arrow). Strip 2 shows a sinus tachycardia and depression of the T wave, 5 minutes after the subcutaneous administration of 0.14 mg of isopropylepinephrine. Strips 3, 4, 5, 6, and 7, taken 7, 10, 15, 20 and 25 minutes after the administration of the drug. Carotid sinus pressure (arrow) fails to produce a standstill due to the development of beats arising from the sinus node and occasional beats arising from a ventricular focus (strips 3 and 4). There is a definite lowering of the diastolic pressure.

for the production of ventricular fibrillation by epinephrine during hydrocarbon inhalation.

The effect of isopropylepinephrine on induced cardiac standstill was studied in 14 patients. The procedure was as follows: an electrocardiogram was made showing the cardiac standstill induced by the carotid sinus compression. A blood pressure reading was also made at this time. Isopropylepinephrine was administered subcutaneously in doses of 0.14 to 0.2 mg. Electrocardiograms showing the effect of carotid sinus pressure were made and blood pressure readings recorded, starting two minutes after the administration of the isopropylepinephrine and thereafter at 1 and 2 minute intervals.

Results. The cardiac inhibition induced by the carotid sinus pressure was abolished in every instance following the administration of isopropylepinephrine (Fig. 1 and 2). This effect was noted within 5 minutes after the injection of the drug. A sinus tachychardia was a constant effect. Changes in the contour of the electrocardiogram were frequent, with elevation and occasional flattening and inversion of the T wave. The standstill was abolished by the restoration of the activity of the sinus node or by the initiation of ectopic auricular or ventricular pacemakers. In some instances, multiple rhythmic foci were induced by the drug. The blood pressure responses were as follows: the systolic pressure was unchanged in 6 instances, slightly elevated

17020

Experimental Gastric and Duodenal Ulcer.

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From a review of the literature concerning peptic ulcer one receives the impression that great caution should be exercised in drawing conclusions from the results of experiments in which ulcers have been produced. Although ulcers can be produced readily in certain animals, the conditions necessary to do this are artificial and alter seriously the normal anatomical and physiological relationships of the organs involved. Usually these alterations are far different from any that could possibly occur in human subjects with peptic ulcer. Species differences, too, constitute a hazard in comparing experimental ulcer in animals with spontaneous ulcer in man.

The evidence tends to show that the two outstanding factors in the formation of experimental gastric and duodenal ulcers are the digestive action of acid gastric secretion, and the lowering of mucosal resistance due to local vascular abnormality. The most successful experimental methods for the production of ulcers utilise one or both of these factors.

It is evident that subacute or chronic ulcers resembling those in man are very difficult to produce. While chronic ulcers appear after the establishment of surgical duodenal drainage,[1] it should be realized that this operation has other profound effects, due to the short-circuiting of bile, pancreatic and duodenal secretion past the greater part of the small intestine.

Chronic combined histamine and nitroglycerine stimulation[2,3] is one of the few experimental methods for the production of gastric and duodenal ulcers utilizing the factors both of acid-peptic digestion and of local tissue ischemia in the intact animal. The reported high incidence of lesions in rabbits caused by this technic suggested it to be dependable in an animal that is refractory to other methods.

Code and Varco introduced the method of chronic histamine stimulation. It consists in embedding histamine in a mixture of beeswax and mineral oil before intramuscular injection. This mixture prolongs the action of the contained histamine by delaying its absorption. In this way a large dose of histamine may be injected intramuscularly, with a resultant chronic effect lasting 24 hours or longer. One intramuscular injection daily is, therefore, sufficient to maintain continuous stimulation of gastric secretion.

It was desired, in our laboratory, to use an efficient method for the production of gastric and duodenal ulcers in rabbits. Reports from Wangensteen's laboratory indicated that it was difficult or impossible to provoke ulcers or erosions in rabbits by the chronic action of histamine alone.[3] However, Baronofsky and Wangensteen[4] were successful in producing ulcers in rabbits by means of chronic combined histamine and nitroglycerine stimulation. They reported erosions or ulcers in 9 of 12 rabbits within an experimental period of 6 days. They explained the occurrence of these lesions on the basis of local tissue ischemia (due to the chronic action of nitroglycerine) which rendered the mucosa more susceptible to the digestive action of a highly acid gastric secretion (caused by the chronic action of histamine). In our experiments a longer period was used, with the hope that a higher incidence of ulcers would occur.

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[1] Mann, F. C., and Williamson, C. S., *Ann. Surg.*, 1923, 77, 409.

[2] Code, C. F., and Varco, R. L., PROC. SOC. EXP. BIOL. AND MED., 1940, 44, 475.

[3] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg. Gynec. and Obstet.*, 1942, 75, 170.

[4] Baronofsky, I. D., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1946, 62, 127.

TABLE I.
Effect of Isopropylepinephrine on Ventricular Rate in Complete Heart Block.

Patient	1	2	3	4	5
Initial ventricular rate	54	38	46	31	20
After admin. of Isopropylepinephrine (min.)					
5	57	38	50	37	27
10	61	43	52	48	32
15	60	47	71	54	45
30	57	68	65	50	49
45	60	60	60	46	45
60		50	54	41	44
75			55		
90		38	52		
105			48		
120		38	47		
Max. increase in ventricular rate	7	30	25	23	29

It is probable that isopropylepinephrine has another important advantage in the treatment and prevention of cardiac standstill. In comparing the results of the present study on carotid sinus induced cardiac standstill with previous observations in which epinephrine was used, it appeared that the pacemaker induced by isopropylepinephrine was usually in the sinus node, in lower auricular foci or in the auriculo-ventricular node. There was seldom an excitation of lower ventricular foci. In contrast, epinephrine frequently induced rhythmic foci from lower ventricular centers, and at times multifocal ectopic ventricular beats resembling a pre-fibrillation rhythm occurred. In this connection the recent report of Garb and Chenoweth[5] is of interest. These observers produced ventricular fibrillation consistently in cats during hydrocarbon inhalation by the administration of epinephrine and norepinephrine, while isopropylepinephrine did not induce this arrhythmia under the same conditions. The sudden cardiac failure which occurs in heart block and during surgical operations may be the result of either cardiac standstill or ventricular fibrillation. It is frequently impossible to determine which of these mechanisms is the basis for the sudden cessation of effective cardiac action. The administration of epinephrine, if transient ventricular fibrillation is the mechanism would tend to perpetuate this arrhythmia leading to a fatal termination. It is also known that the treatment of cardiac standstill by the intracardiac administration of an effective dose of epinephrine may lead to ventricular fibrillation. The availability of a potent compound which does not predispose to ventricular fibrillation should reduce greatly the danger of the administration of a sympathomimetic drug in the therapy of sudden cessation of cardiac activity.

Conclusions. Isopropylepinephrine, a nonpressor homologue of epinephrine increases cardiac rhythmicity, as indicated by the abolition of the carotid sinus induced cardiac standstill, and by an increase of the ventricular rate in complete heart block. This drug may be of practical value in the therapy and prevention of sudden cardiac standstill.

The hydrochloride of isopropylepinephrine known as "Isuprel" was used in this study.

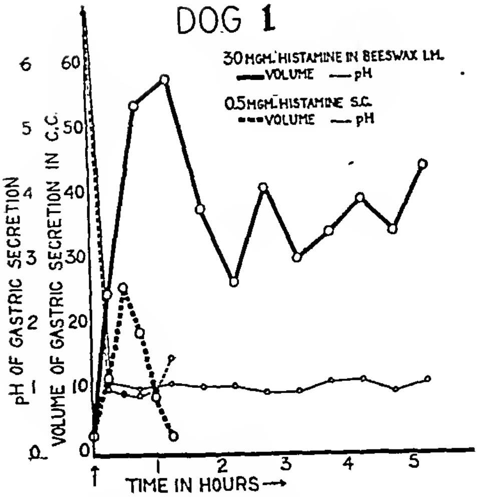

Fig. 1.

Gastric secretory response of dog No. 1. The arrow indicates the time of injection of histamine. Certain volumes are recorded as 15-minute volumes and others as 30-minute volumes.

experiment are plotted on the same graph to emphasize the contrast between the gastric secretory response to a subcutaneous injection of 0.5 mg of histamine in aqueous solution and the prolonged response to an intramuscular injection of 30 mg of histamine embedded in beeswax and mineral oil mixture. Fig. 2 shows the results of similar experiments, using Dog No. 2.

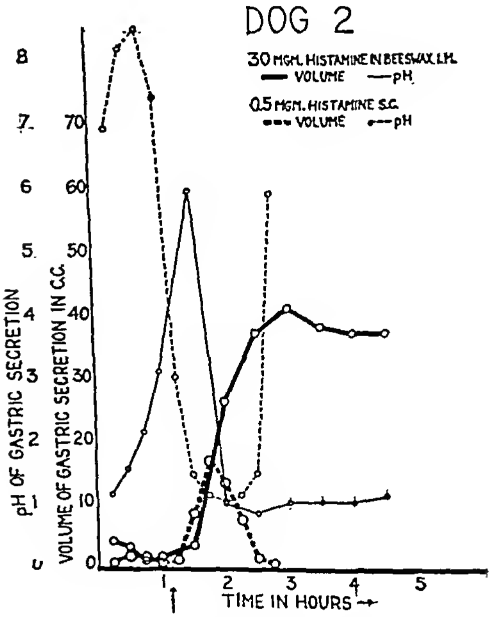

Fig. 2.

Gastric secretory response of dog No. 2. The arrow indicates the time of injection of histamine. Certain volumes are recorded as 15-minute volumes and others as 30-minute volumes.

Following the intramuscular injection of 30 mg of histamine in beeswax and mineral oil our animals showed no evidence of toxic effects of the drug. This was taken as evidence that the large dose of histamine was not absorbed rapidly. In both experiments (Dog No. 1 and Dog No. 2) the rate and acidity of gastric secretion continued at high levels, showing little change in the first few hours. In Dog No. 1 the specimen of gastric secretion obtained 24 hours after the injection still showed a large volume and high acidity of gastric secretion.

These results were taken as proof of the prolonged effects of intramuscular injection of histamine in beeswax and mineral oil. The nitroglycerine control series (6 rabbits) yielded the following results. Two rabbits died of pneumonia during the injection period, one on the 8th and one on the 10th day. Four survivors were sacrificed on the 15th day. Autopsy revealed no gastric or duodenal lesions. This finding was taken as evidence that the chronic action of nitroglycerine alone is incapable of producing ulcerations within a period of 15 days.

Results summarizing the findings in Series I are shown in Table I. In this series, involving 32 rabbits, 12 died during the experimental period. Of these, 9 had perforation of the fundus of the stomach, one had perforation of the first part of the duodenum, one had hemorrhage into the gastrointestinal tract, and one died without a demonstrable lesion to account for its death. Of the 20 rabbits that survived until their sacrifice at the end of the experimental period, 19 had no demonstrable lesion and one had a recent small perforation of the first part of the duodenum with associated localised peritonitis. All the rabbits in this series lost weight during the experiment, so that some were emaciated at the time of death or sacrifice, although food was found in all their stomachs at autopsy.

All perforations of the fundus of the stomach occurred within the first 9 days of the

There are conflicting reports[5-7] in the literature concerning the development of gastric and duodenal ulceration following bilateral vagotomy in experimental animals, particularly rabbits. Some investigators have observed the development of ulcerative lesions in more than 30% of vagotomised rabbits, the incidence increasing progressively with the interval of time after vagotomy. Our results in experiments of a similar nature are reported herein. In addition, we investigated the type and incidence of ulcers of the stomach and duodenum of vagotomised rabbits, provoked by the chronic combined sitmulation of histamine and nitroglycerine.

Materials and methods. In order to demonstrate that our beeswax and mineral oil mixture (prepared according to the method of Code) effectively prolonged the action of histamine, experiments were carried out on 2 dogs, one with a Spivack gastrostomy and the other with a metal gastric fistula. The animals were conditioned to the laboratory so that their fasting secretion was minimal. Control specimens were taken, and 0.5 mg histamine injected subcutaneously. The secretion was collected every 15 minutes and volume and pH determined. Subsequently, in each animal, 30 mg of histamine embedded in beeswax and mineral oil was injected intramuscularly. Specimens were collected every 30 minutes for as long as 5¼ hours and the volume and pH of each determined.

A control series of experiments, using 6 rabbits, was performed to determined whether or not daily intramuscular injections of nitroglycerine, embedded in beeswax and mineral oil, produce gastric or duodenal lesions. These rabbits received the stock diet of Purina rabbit pellets, carrots and water. Daily intramuscular injections of 1 mg of nitroglycerine in beeswax and mineral oil were given. Injections were continued for 14 days in survivors and these rabbits were sacrificed on the 15th day for gross and microscopic examination of the involved organs.

It was felt that Code and Varco had demonstrated amply that injections of beeswax and mineral oil alone do not stimulate gastric secretion or produce ill effects in animals. Therefore, no control series of experiments was carried out to confirm this point.

Series I included 32 adult rabbits maintained on the stock laboratory diet. In this series were investigated the type and incidence of lesions of the stomach and duodenum of rabbits produced by the chronic combined action of histamine and nitroglycerine. Daily administration to each rabbit of 30 mg of histamine and 1 mg of nitroglycerine was carried on until death of the animal or until 14 daily injections had been given. Each drug was embedded in beeswax and mineral oil mixture and injected intramuscularly early in the afternoon. Those animals which died during the experimental period were examined as soon as possible, while all survivors were examined on the 15th day.

Series II included 12 adult rabbits in which investigation was made of the incidence of lesions of the stomach and duodenum following bilateral subdiaphragmatic vagotomy. In performing the vagotomy at least 2.0 cm of esophagus was completely cleared of surrounding tissues down to its muscular layer to ensure division of all fibers of both vagus nerves. As a rule these rabbits completely recovered from the effects of the anesthetic within a few hours and were soon lively and eating. Those animals which died were examined as soon as possible after death. The remaining rabbits were sacrificed at different intervals up to 47 days from the time of operation. Survivors were killed by a blow on the neck and autopsies were performed at once.

In Series III 19 rabbits were vagotomised. After a few days they were given daily intramuscular injections of histamine and nitroglycerine, each embedded in beeswax and mineral oil mixture. Injections were carried on for 15 days in survivors, which were sacrificed on the 16th day.

Results and discussion. Fig. 1 shows the results of the experiments performed using Dog No. 1. The results of both parts of the

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[5] Beazell, J., and Ivy, A. C., *Arch. Path.*, 1936, **22**, 213.

[6] Ophuls, W., *J. Exp. Med.*, 1906, **8**, 181.

[7] Alvarez, W. C., Hosoi, K., Overgard, A., and Ascanio, H., *Am. J. Physiol.*, 1929, **90**, 631.

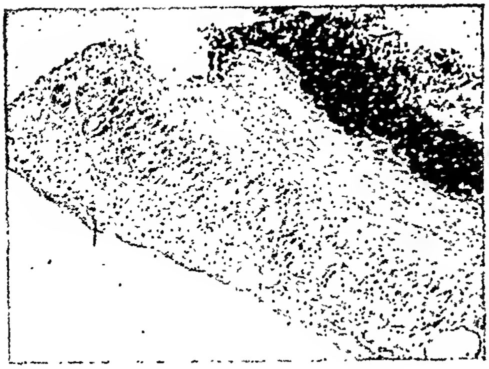

Fig. 3.
Photomicrograph: Rabbit 20, Series I. This shows a section of gastric wall at the edge of the perforation of the fundus. The mucosa shows superficial necrosis, with numerous shallow erosions. The muscularis mucosae and the submucosa are both edematous, so that there is great thickening of this zone of the wall. The submucosa is exposed for a short distance adjacent to the perforation. The muscularis is somewhat edematous and shows a narrow zone of necrosis at the edge of the perforation. The subserosal tissue is edematous and the serosa is necrotic and detached for a short distance. The blood vessels in the submucosa and muscularis are markedly engorged with blood, and the veins, particularly are greatly dilated. There is absence of inflammatory cell infiltration and there is no sign of a repair process.

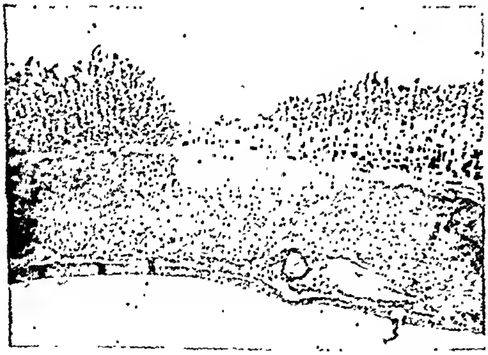

Fig. 4.
Photomicrograph: Rabbit 21, Series I. This shows a section of a gastric mucosal erosion near a perforation of the fundus. There are scattered superficial erosions of necrotic areas of mucosa. The submucosa, muscularis and serosa appear relatively normal apart from marked engorgement and dilatation of the subserosal blood vessels. No apparent inflammatory cell infiltration or evidence of repair is present about the bases of the erosions.

the animal, the other at the time of sacrifice (Fig. 5), were quite similar to one another in microscopic appearance. Sections were cut so as to pass through the involved duodenal wall immediately adjacent to the perforation. There was a moderate degree of acute inflammatory cell infiltration (polymorphonuclear leucocytes and lymphocytes) in the wall of the duodenum surrounding the perforated ulcer. In places there were focal collections of lymphocytes, particularly in the mucosa and submucosa, which were considered to be lymphoid follicles and not part of the inflammatory reaction. The wall of the ulcer contained necrotic tissue with a somewhat homogeneous, eosinophilic appearance. In one instance a thrombosed submucosal artery was seen in the wall of the perforated duodenal ulcer. The ulcer crater was cone-shaped, wider in diameter at the mucosal side of the wall. The duodenal wall at a distance from the perforated ulcer appeared essentially

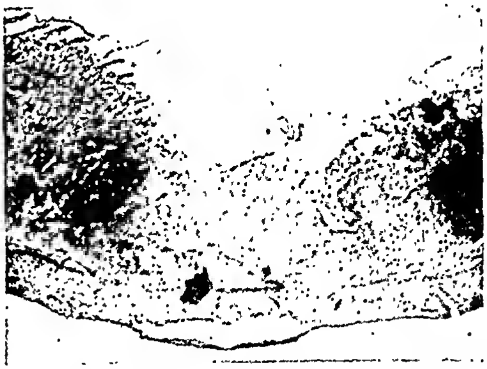

Fig. 5.
Photomicrograph: Rabbit 25, Series I. This is a tangential section through the wall of a perforated duodenal ulcer showing the tissues immediately adjacent to the actual perforation. On each side of the perforation, a short distance away, the wall of the duodenum appears essentially normal. Occasional collections of lymphocytes are present in the mucosa and submucosa representing naturally-occurring follicles and not part of an inflammatory process. The tissue immediately adjacent to the perforation is necrotic, the zone being wider towards the mucosal side and narrower towards the serosal surface. A mass of poorly-stained necrotic debris is lying semi-detached at the level of the mucosa and submucosa. The continuity of the muscularis is seen in this section near the perforation but it is necrotic and no cellular detail is visible. The serosa is necrotic and partially detached. There is a zone of edema of all layers near the necrotic area and a very few polymorphonuclear leucocytes are to be seen in the submucosa at this point. No definite evidence of repair can be detected.

TABLE I—Series I.

Rabbit	No. daily inj. of histamine and nitroglycerine	Died	Sacrificed	Gross autopsy findings
1	3	X		Perforation, fundus of stomach
2	11		X	No lesion
3	11		X	” ”
4	11		X	” ”
5	6	X		Perforation of fundus and blood in bowel
6	7	X		Perforation of fundus
7	8	X		Perforation of fundus and blood in bowel
8	14		X	No lesion
9	14		X	” ”
10	14		X	” ”
11	14		X	” ”
12	14		X	” ”
13	14		X	” ”
14	14		X	” ”
15	14		X	” ”
16	14		X	” ”
17	14		X	” ”
18	1	X		Perforation of fundus of stomach
19	2	X		Perforation of fundus
20	3	X		” ” ”
21	3	X		” ” ”
22	5	X		” ” ”
23	11	X		No lesion
24	12	X		Intestinal hemorrhage, no lesion found
25	13	X		Perforation of duodenum
26	14		X	No lesion
27	14		X	” ”
28	14		X	” ”
29	14		X	” ”
30	14		X	” ”
31	14		X	Perforation of duodenum
32	14		X	No lesion

experiment; namely, after 1, 2, 3, 3, 3, 5, 7 and 8 injections, respectively. The short time required for the production of these lesions, as well as their microscopic appearances indicate that they were in every sense acute. The perforations of the duodenum, one fatal after 13 daily injections and the other discovered after 14 daily injections, required a somewhat longer time to develop. They were much smaller in size than the gastric perforations.

Generally speaking, the gastric wall near the site of perforation showed very little inflammatory cell infiltration and only moderate edema. All layers of the gastric wall appeared relatively normal to within a short distance of the edge of the perforation, except for scattered superficial mucosal erosions in several instances. The muscularis, at the site of perforation, came to an abrupt end with a small zone of necrosis. The mucosa was absent over a narrow zone about the perforation, leaving the submucosa exposed. This may have been due to retraction of the mucosa after perforation occurred. In some instances minimal leucocytic infiltration was noted, chiefly in the submucosa. Frequently great dilation of blood vessels in the submucosa or subserosal zone was seen.

The preceding brief description of the appearance of sections of gastric wall adjacent to a perforation may be considered representative of the findings in all nine instances of gastric perforation. The gross and microscopic appearances (Table I, Fig. 3, 4) together with the rapid development of these lesions, suggest an acute necrosis of all layers of the gastric wall with digestion of the necrotic tissue resulting in the large, ragged type of perforation. Acute peritonitis, due to the escape of gastric contents into the peritoneal cavity, with or without hemorrhage amply explains the fatal issue in these rabbits.

The two perforations of the first part of the duodenum, one discovered after death of

TABLE III—Series III.

Vagotomised rabbit	No. daily injections	Died	Sacrificed	Autopsy findings
1	15		X	No lesion
2	15		X	’’ ’’
3	0	X		Pneumonia
4	15		X	No lesion
5	15		X	’’ ’’
6	15		X	’’ ’’
7	15		X	’’ ’’
8	15		X	’’ ’’
9	0	X		Pneumonia
10	15		X	No lesion
11	15		X	’’ ’’
12	15		X	’’ ’’
13	0	X		(Anesthetic death)
14	0	X		(No autopsy performed)
15	0	X		’’ ’’ ’’
16	2	X		Infection—ear
17	5	X		No lesion (emaciation)
18	5	X		Pneumonia
19	6	X		No lesion (emaciation)
20	6	X		Pneumonia
21	9	X		’’
22	9	X		No lesion (emaciation)
23	12	X		Pneumonia
24	14	X		No lesion (emaciation)

4 deaths could not be accounted for by any demonstrable lesion apart from marked emaciation. Autopsies revealed no gastric or duodenal ulcerative lesions in any animal in this series of experiments. Microscopic examination of the stomach and duodenum of the animals in this series revealed no abnormalities.

Conclusions. 1. Daily intramuscular injections of 1 mg of nitroglycerine embedded in a mixture of beeswax and mineral oil does not provoke gastric or duodenal ulceration in rabbits within a period of 15 days.

2. Bilateral subdiaphragmatic vagotomy does not produce gastric or duodenal ulceration within periods as long as 47 days in rabbits maintained on a diet of Purina rabbit pellets, carrots and water.

3. Daily intramuscular injections of histamine (30 mg) and nitroglycerine (1 mg) embedded in a mixture of beeswax and mineral oil produce acute, necrotic, perforating lesions of the stomach or duodenum of approximately 30% of rabbits. These lesions are fundamentally different from typical human peptic ulcers.

4. Bilateral subdiaphragmatic vagotomy apparently exerts a measure of protection against the development of acute ulcerative lesions of the stomach and duodenum of rabbits produced by the chronic action of histamine and nitroglycerine.

5. Gastric and duodenal ulcers produced in rabbits by chronic combined histamine and nitroglycerine stimulation are not considered to be suitable lesions for the experimental assessment of ulcer prevention measures.

TABLE II—Series II.

Rabbit	Survival time, days after operation	Died	Sacrificed	Autopsy findings
1	47		X	No lesion
2	47		X	” ”
3	32	X		Pneumonia
4	20	X		Ear infection
5	43		X	No lesion
6	18	X		Infected wound
7	5	X		Pneumonia
8	38		X	No lesion
9	31	X		Pneumonia
10	31		X	No lesion
11	25	X		Pneumonia
12	4	X		”

normal. No definite evidence of repair was detected in the sections so that these lesions were considered to be acute.

Gross and microscopic examination of the small perforations of the first part of the duodenum suggested the process to have been due to infarction of an area of the duodenal wall followed by the development of an acute perforating ulcer. Although acute in appearance the lesion had developed slowly enough to permit inflammatory cell infiltration about it. This may be contrasted with the large, rapid perforations of the fundus of the stomach which developed in other animals.

In the 21 animals showing no gross lesions of the stomach or duodenum at autopsy, the microscopic appearance of sections of the gastric and duodenal wall was essentially normal.

Table II summarizes the findings in the 12 experiments in Series II. None of the rabbits in this series developed lesions of the stomach or duodenum within periods varying from 4 to 47 days following vagotomy. Seven of the 12 animals died after vagotomy, on the 4th, 5th, 18th, 20th, 25th, 31st and 32nd day, respectively. Of these, 5 died of pneumonia, one of a wound infection and one of an ear infection. The remaining 5 were sacrificed on the 31st, 38th, 43rd, 47th, and 47th days, respectively, after vagotomy.

At autopsy the stomach of each of these animals was found stuffed with food and appeared definitely larger with greater intragastric pressure than was noted during autopsies upon non-vagotomised rabbits. The esophagus was empty in each instance, while the duodenum was either empty or contained a small quantity of bile-stained fluid. No gross lesions were found in other organs apart from those mentioned previously as a cause of death. In each case, the microscopic appearance of the stomach and duodenum was essentially normal. The incidence of pneumonia in this series was notably greater than in animals not subjected to vagotomy. Vagotomy may permit aspiration pneumonia from regurgitation of gastric contents.

It would appear, from the results of this series of experiments, involving 12 vagotomised rabbits, that gastric or duodenal ulcers are not likely to develop following this operation within periods of up to 47 days.

Results summarising the findings in Series III are shown in Table III. Although 24 rabbits were vagotomised in this series, 5 died before the injections of histamine and nitroglycerine were begun. Two rabbits died of pneumonia; one did not recover consciousness following the operation; and the causes of death of the other two are unknown as no autopsies were performed upon them. The three rabbits examined did not have lesions of the stomach or duodenum at the time of death.

Since 5 rabbits died before the injections were begun, it was necessary to exclude them; so that this series actually consisted of nineteen vagotomised rabbits injected daily as described. Of these, nine died before the 16th day of the experiment, at which time the survivors were sacrificed for examination. Of the 9 deaths, 4 were caused by pneumonia, one by infection of an ear, while the other the course of development of the protamine titration. It was found that prothrombin deficient and thrombocytopenic bloods were more sensitive than normal bloods to the effects of added heparin, both *in vitro* and *in vivo*. In this connection the observations of Howell are of interest.[5]

He observed that the effects of amounts of heparin just sufficient to prevent clotting could be overcome by cephalin obtained from brain tissue, recognizing this as a substance also found in platelets. It is possible that other factors may also influence both *in vitro* and *in vivo* heparin sensitivity.

It is doubtful if the increases in the protamine titration can be explained by thrombocytopenia alone. The protamine titration may be normal in severe thrombocytopenia and may be markedly increased in patients with normal platelet counts and prothrombin times.

In order to produce an increase of 0.020 mg in the protamine titration (the smallest increment of increase used) 0.018 mg of liquid beef heparin (Abbott) per ml of human blood was required. This was 2 to 5 times the amount of heparin required to increase the clotting time of whole blood or plasma *in vitro*, and was 25- to 50-fold greater than that required for "platelet-free" plasma.[4] These comparisons were made on normal human blood and plasma and do not necessarily apply to bleeding disorders. Moreover, it has been observed that animals on aminopterin develop an increased protamine titration without thrombocytopenia or an increased *in vitro* heparin sensitivity.[7]

The protamine titration, however, is affected by several factors, most of which influence fibrin formation. Among these factors are: heparin or heparin-like substances, prothrombin deficiency, impairment of the conversion of prothrombin to thrombin, hemophilia, and the absence of fibrinogen. It is possible to set up a very sensitive protamine titration over a narrow range of protamine concentration which can be altered by changes in the platelet count but such a titration would lose its clinical value. The problem of the effect of thrombocytopenia appears to be sufficiently controlled that the results of the protamine titration[1] must be explained on some basis other than platelet deficiency alone.

Twenty-one patients, representing a variety of malignant and benign disorders, had normal platelet counts ranging from 180,000 to 324,000 per cmm but showed mild to marked increase in the protamine titration (0.16 mg to 0.40 mg). On the other hand, eighteen patients with similar bleeding disorders had moderate to marked thrombocytopenia (20,000 to 108,000 platelets per cmm). In these the protamine titration was normal. These observations were repeated 6 to 9 times on some patients. Most patients who had an increased protamine titration also had thrombocytopenia of some degree. In those who responded to toluidine blue the platelet count, before and after partial or complete control of bleeding occurred, was not appreciably altered, Table I.

In Werlhof's syndrome three patients had platelet counts ranging from 2,000 to 60,000 and normal protamine titrations. These did not respond to toluidine blue. Three other patients with platelet counts from 23,000 to 60,000 per cmm and increased protamine titrations (0.16 to 0.20 mg), showed some decrease in bleeding as their titrations returned to normal, but all 3 required splenectomy for complete control of bleeding. In only one of these was the response to toluidine blue impressive. The detailed studies on these and other patients are presented elsewhere.[2]

Comment. These data indicate that the protamine titration may be increased in the presence of normal platelet counts as well as in severe thrombocytopenia. Moreover, the protamine titration can be returned to normal by the administration of adequate toluidine blue without materially altering the platelet count. In addition, thrombocytopenia may

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[5] Howell, W. H., *The Harvey Lectures*, 1917, **12**, 272.

[6] Allen, J. G., Sanderson, M. H., Milham, M., Kirschon, A., and Jacobson, L. O., *J. Exp. Med.*, 1948, **87**, 71.

[7] Grossman, B. J., Sanderson, M. H., and Allen, J. G., to be published.

17021

Independence of Protamine Titration and Platelet Level in Certain Hemorrhagic Diseases.

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Following the addition of a standard quantity of heparin to normal blood, the amount of protamine sulfate required to clot this blood within one hour is remarkably constant.[1] Although species differences do exist the amount of protamine required for members of the same species varies to only a slight degree.

This protamine requirement as determined by the protamine titration[2] may be greatly increased in certain pathologic states associated with hemorrhage. Increased protamine requirements in whole blood of patients have been observed in association with acute leukemia, chronic leukemia, some cases of idiopathic thrombocytopenia, secondary thrombocytopenia, aplasia of the bone marrow, and in the toxic states associated with total body X-radiation, nitrogen mustards and aminopterin therapy. All of these conditions are frequently associated with thrombocytopenia. The hemorrhagic complications of these disorders are often temporarily improved or controlled and the protamine titration returned to normal by the administration of toluidine blue, even though the platelet count is unchanged. An increased protamine titration has also been found in bleeding patients with normal platelet counts and normal prothrombin activity.[2] These patients likewise respond to toluidine blue and protamine therapy, and the protamine titrations return to or toward normal.

These and other observations suggest that the increased protamine titration may be due to a defect in the clotting mechanism similar although not identical with that produced by the intravenous injection of commercial heparin. We have been able to demonstrate certain differences between this heparinoid defect and that produced by the intravenous injection of commercial beef heparin in both man and dog.[3] The principal difference appears to be in anticoagulant potency. The bloods of patients and dogs with these disorders rarely inhibited or delayed coagulation of normal bloods. Furthermore, the protamine titration was increased in many of our patients and experimental animals to such an extent that had the increased protamine titration resulted from heparinemia identical with that produced by an injection of commercial beef heparin (Abbott), the blood would invariably have been incoagulable. Incoagulable blood was found in only a few instances. Generally, the clotting time was prolonged only 2 to 4 times the normal values.

Recently Conley, Hartmann and Lalley[4] have observed that normal human plasma could be made more sensitive to the effects of added heparin by removing most of the platelets by centrifugation. They concluded that these plasma data were applicable to the protamine titration and stated that the protamine titration "can be explained by the variation in the platelet concentration alone." The protamine titration, however, is performed on whole blood.

Observations of the type reported by the Hopkins group[4] were investigated by us in

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[1] Allen, J. G., Moulder, P. V., Elghammer, R. M., Grossman, B. J., McKeen, C. L., Sanderson, M. H., Egner, W. M., and Crosbie, J., *J. Lab. and Clin. Med.*, 1949, **34**, 473.

[2] Allen, J. G., Grossman, B. J., Elghammer, R. M., Moulder, P. V., McKeen, C. L., Jacobson, L. O., Pierce, M., Smith, T. R., and Crosbie, J., *S.G.O.*, in press.

[3] To be published.

[4] Conley, C. L., Hartman, R. C., and Talley, J. S., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 284.

17022

Influence of Benzyl-Imidazoline on the Peripheral Circulation of Man.*

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Studies have shown that 2-benzyl-4,5-imidazoline hydrochloride (Priscol hydrochloride-Ciba) has certain properties which may be useful in augmenting the volume of blood flow to the periphery in man.[1] It reverses the hypertensive effect of epinephrine to a hypotensive one through an adrenolytic action.[2] Following the use of this drug in animals, peripheral vasodilatation cardiac stimulation, and an increased cardiac output have been shown.[2] The present study was carried out to determine the effects of the intravenous injection of this drug upon the peripheral circulation in normal individuals and patients with arteriosclerotic obliterative disease and thromboangiitis obliterans.

Methods and materials. Seventy male subjects without evidence of peripheral vascular disease, between the ages of 18 and 45 (average 26) and 10 subjects with organic obliterative disease, between the ages of 35 and 55 (average 51) were studied. Of the latter 7 had arteriosclerosis obliterans and 3 thromboangiitis obliterans. The volume change of the digits was determined using the Cambridge pneumo-plethysmograph[3] and the venous occlusion method.[4] The right or left second toe tip and right or left index finger tip were enclosed in a digital cup from which volume changes were recorded. The collecting cuff was placed at the wrist or ankle. Venous occlusion was ordinarily accomplished by quickly inflating a ribbed-type blood pressure cuff to a pressure of 60 mm of mercury. Lower pressures were often employed in patients with vascular disease. Plethysmograms were standardized so that volume changes were obtained in cu mm per 5 cc of tissue per second. The amplitude of pulsations was recorded in cu mm per 5 cc of tissue. Normal subjects were used as controls for the patients with organic disease. Also control recordings were taken before and at frequent intervals after the injection of the drug so that each subject acted as his own control. Skin temperatures were obtained from a Micromax automatic 4-point recorder which recorded from each thermocouple junction every 2 minutes. Recordings were also made using a Brown Electronik 4-point potentiometer which recorded from each junction every 30 seconds. The readings were accurate to ± 0.25°C. The accuracy of the instruments was tested using a National Bureau of Standards mercury thermometer which could be read accurately to 0.1°C. The thermocouple junctions were taped to the skin with cellulose tape and inserted into the muscle or subcutaneous tissue of the calf using a specially prepared needle. Determinations were made under standard reproducible conditions. The patients had a normal meal the night before the test. They reported, without breakfast, to the laboratory, dressed in a gown and bathrobe. They rested 45 minutes prior to the test. Their bodies were covered by their robes with no additional blankets during the test (except as described below). The determinations were made in a closed room without draughts. The room temperature did not vary more than ± 1.5°C during an experiment. Indirect heating of the subjects was produced by covering them with 2 wool blankets (the finger tips and toe tips were exposed)

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* The opinions held are those of the authors and not necessarily those of the Veterans Administration.

[1] Chess, D., and Yonkman, F. F., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 127.

[2] Ahlquist, R. P., Huggins, R. A., and Woodbury, R. A., *J. Pharmacol. and Exp. Therap.*, 1947, **80**, 271.

[3] Burch, G. E., *Am. Heart J.*, 1947, **33**, 48.

[4] Goetz, R. H., *Am. Heart J.*, 1946, **31**, 146.

TABLE I.
Patients with Increased Protamine Titrations and Low Platelet Counts Before and After *Receiving Toluidine Blue.*

Patient	Diagnosis	Platelets		Protamine titration*	
		Before toluidine blue, 1000 per cmm	After toluidine blue, 1000 per cmm	Before toluidine blue, mg protamine sulfate	After toluidine blue, mg protamine sulfate
453730	Acute leukemia	190	17	.18	.14
K-a234	” ”	20	80	.18	.16
413730	” ”	52	24	.18	.16
436363	Acute leukemia Aminopterin Rx	140	24	.18	.16
341183	Hodgkin's Disease Nitrogen Mustard Rx	124	37	.20	.16
395060	” ”	83	85	.20	.14
409460	” ”	64	37	.20	.14
435361	” ”	81	75	.16	.14
453179	” ”	19	70	.16	.14
M-a186	Chronic leukemia Spray irradiation	10	16	.18	.16
423054	Cancer Breast P^{32} therapy	100	93	.18	.16
T-a229	Toxic Pancytopenia	7	16	.18	.14
15517	Toxic depression of bone marrow	85	15	.18	.14
H-b32	Menorrhagia	66	72	.16	.14

* Following the addition of a standard concentration of heparin to normal blood a remarkably constant amount of protamine is required to restore clotting. (Laboratory normal is 0.140 mg protamine sulfate (Lilly) per ml blood containing 0.091 mg heparin (Abbott)).

occur without increasing the protamine titration.

The protamine titration is probably increased by factors which directly or indirectly delay or inhibit fibrin formation. Abnormal values in the protamine titration can not be interpreted unless all known clotting factors can be evaluated. The titration can be increased when large quantities of heparin or heparin-like substances are present, but it does not necessarily follow that the factor(s) responsible for the increased protamine titration in these patients was actually heparin or even heparin-like. Some endogenous compounds, not of the heparin family, were found to be mild anticoagulants and were inhibited by both toluidine blue and protamine sulfate.[3] The exact nature of the factor(s) responsible for the defect observed in these patients remains unknown.

Summary. 1. The platelet concentration influences the sensitivity of bloods and plasmas to the effect of added heparin. The anticoagulant potency of heparin is also enhanced by prothrombin deficiency. The potentiated effect of heparin in thrombocytopenic bloods may be due to reduced thromboplastin (cephalin) activity.

2. The protamine titration may be increased when the platelet count is normal and it may be normal in the presence of thrombocytopenia. It is influenced by heparin, heparinoid substances, prothrombin deficiency, hemophilia, and possibly other factors.

3. Increased protamine titrations may occur in bleeding patients who have no other apparent clotting defects, including the whole blood clotting time. Many of these patients cease bleeding when given intravenous toluidine blue. The protamine titration under these conditions can be returned to or toward normal regardless of the platelet level.

imidazoline produced a definite increase in skin temperature which was greatest on the large toe (from 29.5°C to 33.25°C in 56 minutes). The increase in temperature of the skin of the calf, subcutaneous and intramuscular tissue of the calf was less. Indirect heating, after benzyl-imidazoline, produced a further increase in the temperatures, the change being greatest in the large toe. After immersing the arm in water 45°C, the temperature of the large toe rose to 34.4°C. Thus, indirect heating of one arm following benzyl-imidazoline augmented the skin flow to the lower extremity.

Effect of benzyl-imidazoline on skin temperatures following indirect heating. Ten normal subjects were studied. The room temperature averaged 24°C. After 20 minutes of indirect heating the average skin temperature of the dorsum of the left foot was 34.6°C, indicating maximum skin temperature effect ordinarily produced by this procedure. Thirty-two minutes after benzyl-imidazoline the temperature of the foot was 34.7°C. Thus, benzyl-imidazoline after indirect heating had no significant effect on the skin flow to the dorsum of the foot.

The duration of toe temperature changes following benzyl-imidazoline was determined in 10 normal subjects whose resting skin temperatures were less than 32°C. The average increase in temperature was 1.5°C in the first 8 minutes and maximum increase 3°C with a beginning decrease 50 minutes after injection.

Discussion. Benzyl-imidazoline intravenously was effective in increasing the volume of the digits and elevating the skin temperature of the lower extremities in normal individuals and in patients with arterial obliterative disease. The volume changes produced by benzyl-imidazoline were only ¼ as large as the volume changes produced by indirect body heating or arterial occlusion for 5 minutes. It is possible that the intra-arterial injection of this drug would be more effective if tissue fixation is high. The greatest increase in temperature was recorded from the skin of the toe. This effect on skin temperatures occurred within 8 minutes after intravenous injection and after approximately 50 minutes a beginning diminution in effect was noted. A greater increase in temperature and blood flow would probably have been recorded if subjects had been in a state of vasoconstriction prior to the administration of benzyl-imidazoline. The more superficial tissues of the lower extremities showed the greatest elevation in temperature. The temperatures of the rectum, forehead, finger, muscles and subcutaneous tissues of the calf changed relatively little. Benzyl-imidazoline was not effective in raising the skin temperature of the extremities after vasodilatation had been produced by indirect heating. Certain patients with arteriosclerotic obliterative disease responded to the drug with an increased skin flow to the periphery.

In these experiments toxic effects were few. The drug was administered very slowly and was discontinued if signs of toxicity were noted. On two occasions benzyl-imidazoline shock developed with a decrease in rate of blood flow to the periphery, with pallor, sweating and weakness.

The increase in rate of blood flow to the lower extremities is probably brought about by more than one mechanism. Peripheral vasodilatation and cardiac stimulation in animals have been shown.[2] In some of its actions, benzyl-imidazoline resembles epinephrine; in others, histamine or acetylcholine; and still others, ergotamine. Vasodilatation was not prevented by atropine or by sympathomimetic agents and therefore probably was not due to acetylcholine-like or histamine-like action. The sympatholytic action is shown by the effective blocking of the pressor action of epinephrine in experimental animals. Epinephrine following benzyl-imidazoline is an active vasodilator (sympathomimetic anti-pressor action).[4] Benzyl-imidazoline does not prevent the cardiac stimulation produced by epinephrine. Thus, the increased flow in man may be due to peripheral vasodilatation combined with an increased cardiac output.

Summary. Benzyl-imidazoline given intravenously effectively increased the rate of blood flow to the periphery in normal individuals and in certain patients with arteriosclerotic obliterative disease. The volume change of the digits following venous occlusion and the skin temperatures were aug-

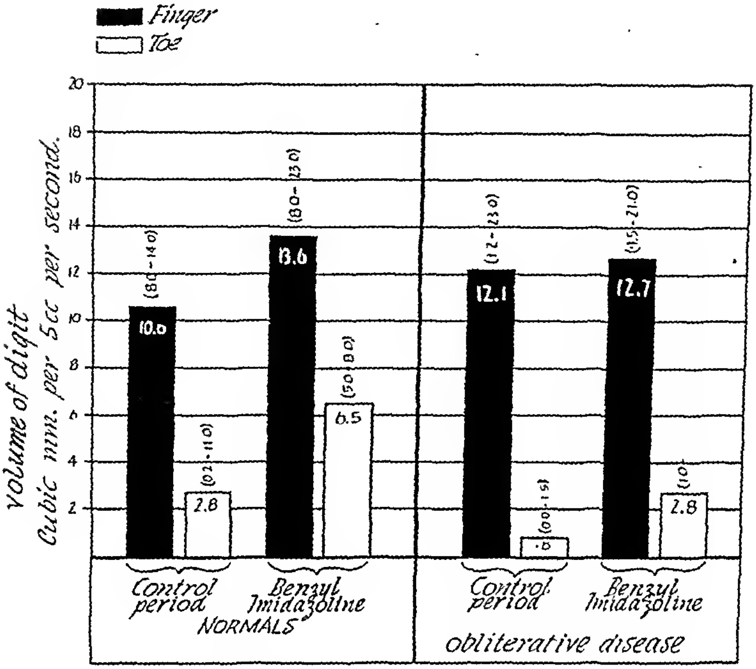

FIG. 1.

Effect of benzyl-imidazoline on the volume of the finger tip and toe tip in 10 normal individuals and 7 patients with arteriosclerotic obliterative disease and 3 patients with thromboangiitis obliterans. The average of 10 determinations was taken on each subject before and 30 minutes after benzyl-imidazoline.

and placing 1 hand and forearm in water at 45°C for 20 minutes. In all experiments 50 mg (5 cc) of benzyl-imidazoline were injected intravenously.

Effect of benzyl-imidazoline on the volume and amplitude of pulsation of the digits. Ten normal subjects and 10 patients with obliterative arterial disease (7 with arteriosclerosis and 3 with thromboangiitis obliterans) were studied (Fig. 1). The room temperature averaged 24°C. In the normal group benzyl-imidazoline increased the volume of the toe tip 1.3 times over that of the control. In the finger tip in this group the change was from 10.6 to 13.6. In patients with obliterative disease the volume of the toe tip increased 2.5 times over that of the control. In the finger tip in this group the change was from 12.1 to 12.7.

Benzyl-imidazoline produced no appreciable change in amplitude of pulsation in the toe tip and finger tip, the values averaging 0.4 before and 0.5 cu mm per 5 cc 30 minutes after in the former and 1.5 and 0.8 cu mm per 5 cc in the latter.

Effect of benzyl-imidazoline on temperatures of deep and superficial tissues. Serial body temperatures were recorded from several body regions before and after injection of benzyl-imidazoline. Ten normal subjects were allowed to rest for 40 to 60 minutes until temperatures varied no more than 0.5°C before the drug was administered. Thirty minutes after injection the temperatures (degrees Centigrade) increased over the resting temperature as follows: forehead 1.0, index finger 1.0, muscle of the calf 0.5, subcutaneous tissue of the calf 0.25, skin of the calf 0.5, skin of the toe 3.75. There was no measurable alteration in the rectal temperature. Thus, benzyl-imidazoline was most effective in increasing the skin flow of blood of the toe.

Effect of indirect heating after benzyl-imidazoline on temperatures of the lower extremity. In 10 normal subjects, at a room temperature which averaged 25°C, benzyl-

TABLE I.
Differential Sheep Cell Agglutination Test Applied to Sera of 160 Patients.

Clinical diagnosis	No.	"Positive"* No.	"Positive"* %	Differential agglutination titer 0-2	4	8	16	32	64	128	256+
Rheumatoid arthritis											
Marked activity	20	13	65.0	6	1	0	3	6	3	1	0
Mild-moderate activity	37	5	13.5	23	4	6	2	3	0	0	0
No activity	21	0	—	19	2	0	0	0	0	0	0
Other arthritis											
Ankylosing spondylitis	10	1	10.0	9	0	0	0	1	0	0	0
Rheumatic fever	5	0	—	5	0	0	0	0	0	0	0
Miscellaneous	15	0	—	14	1	0	0	0	0	0	0
Other diseases											
Visceral angiitis	5	0	—	5	0	0	0	0	0	0	0
Infectious mononucleosis	7	0	—	7	0	0	0	0	0	0	0
Lymphoma (Hodgkin's, leukemia)	8	0	—	8	0	0	0	0	0	0	0
Hepatitis	24	1	4.1	21	1	1	1	0	0	0	0
Lues	8	0	—	8	0	0	0	0	0	0	0

* "Positive": A differential titer of 16 or over.

false results which might be attributable to remaining traces of soap or detergent.

For sera adsorbed with boiled guinea pig kidney and boiled beef erythrocytes the technique of Davidsohn[4] was followed, except that 1 part of serum was mixed with only 3 parts of 20% suspensions of adsorbent. Because of this, some adsorptions were incomplete.

The diagnosis of rheumatoid arthritis was established in patients on the basis of the opinions of several physicians based on typical clinical observations and laboratory findings. Patients were grouped, relative to the activity of their disease, as "marked," "moderate," and "none."

Results. Sera from 160 patients, 78 of whom carried the diagnosis of rheumatoid arthritis were examined. Table I shows their clinical diagnoses and the results of the differential sheep cell agglutination test carried out on their sera. Among the patients with rheumatoid arthritis, one-third of the group with marked activity had titers in the "normal" range, and two-thirds had differential titers of 16 or more. Almost half of the rheumatoid patients were classified as mildly or moderately active. All of them had pain, progressive limitation of motion, joint changes, increased perspiration, an elevated erythrocyte sedimentation rate and often anemia. Their differential agglutination titers extended over a wide range but only in a few patients were they 16 or higher. In the 21 patients with arrested, inactive rheumatoid arthritis the test was uniformly negative. Eighty-two patients comprised the control group as seen in Table I. Six of the 10 cases of ankylosing spondylitis were definitely active. The single case showing a high differential titer did not appear to be different from the others and had no peripheral joint involvement. No other group showed any tendency toward elevated differential titers except a few patients with hepatitis in whom abnormal globulins might be expected. Two patients with titers of 8 and 16 suffered from severe cirrhosis with reversal of the albumin-globulin ratio.

Thus differential agglutination titers of 16 or more were encountered mainly in patients with active rheumatoid arthritis, but only a small proportion of such patients had high titer sera. The impression was gained that high differential titers occurred predominantly in the most severely ill rheumatoid patients.

It was of interest to determine whether the differential sheep cell agglutination test varied with the treatment and clinical course of the disease. In Table II are shown the results obtained with sera of patients on whom tests were repeated over a period of 3-5 months. In mented to a greater degree in the lower extremities than in the upper. The amplitude of pulsation showed less change than did the skin temperature or volume changes of the digits.

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[4] Davidsohn, I., *J. Am. Med. Assn.*, 1937, **108**, 289.

The greatest rise in temperature was seen in the toes. The temperature of the skin of the calf, subcutaneous tissues, and deep muscle of the calf showed a decreasing temperature effect in the order shown. The rectal temperature and temperature of the forehead showed the least change.

Benzyl-imidazoline was not effective in raising the skin temperature of the foot, forehead, or hand after indirect heating of one arm.

Benzyl-imidazoline shock was seen on 2 occasions.

The action of benzyl-imidazoline is complex, and the increased flow to the extremities may result from a combination of effects including peripheral vasodilatation, and, possibly, cardiac stimulation.

17023

Differential Sheep Cell Agglutination Test in Rheumatoid Arthritis.*

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One of the greatest obstacles to the objective study and evaluation of therapy in rheumatoid arthritis has been the lack of a simple and reliable laboratory test for the measurement of rheumatoid activity. Many tests have[1] been proposed in the past and several have proven useful adjuncts to diagnosis and evaluation of a patient but no one test alone is sufficiently significant to serve as sole guide for therapy. Recently Rose, Ragan *et al.*[2,3] have proposed a new simple test for activity in rheumatoid arthritis and have claimed for it a high degree of specificity and reliability. These authors discovered that the serum of patients with rheumatoid arthritis in the active stage contains a substance which agglutinates sheep erythrocytes sensitized with specific amboceptor to much higher titer than normal sheep cells. The test consists in a comparison of the titers to which normal and sensitized sheep cells are agglutinated by patients' serum. The results are expressed as "Differential titer" *i.e.* the algebraic difference between the agglutination titers observed with normal (NS) and sensitized sheep erythrocytes (SS). In patients with active rheumatoid arthritis this differential agglutination titer was found by Rose *et al.* to be "never lower than 16 and usually considerably higher." In other diseases examined (with exception of one case of ankylosing spondylitis) the differential titer was 16 or less.

In order to accumulate data on the validity and possible limitations of this laboratory test, sera from a number of patients were examined in this laboratory. The present paper reports the results from 160 patients living in Northern California, about half of whom had rheumatoid arthritis.

Materials and Methods. The technique described by Rose *et al.*[2] for the differential sheep cell agglutination test was followed in all details. Sera of patients were obtained from freshly drawn venous blood and were tested usually within 18 hours, always within 72 hours of collection. Red blood cells were obtained from a number of different sheep but all gave identical results when tested with the same sera. Special attention was paid to careful preparation of glassware to avoid any

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* Thanks are due to many physicians who cooperated in this study by furnishing patients or sera and offering advice.

[1] Collins, D. H., *Practitioner*, 1948, **161**, 180.

[2] Rose, H. M., Ragan, C., Pearce, C., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 1.

[3] Editorial, *J. Am. Med. Assn.*, 1948, **138**, 514.

TABLE III.
Differential Sheep Cell Agglutination Test Applied to Sera Absorbed with Boiled Beef Erythrocytes and Boiled Guinea Pig Kidney.

Serum	Absorbed with	NS[*]	SS[†]	Differential aggl. titer
Normal human	Unabsorbed	8[‡]	16	2
	Beef erythrocytes	16	16	0
	Guinea pig kidney	0	0	0
Infectious mononucleosis	Unabsorbed	4096	4096	0
	Beef erythrocytes	64	128	2
	Guinea pig kidney	4096	4096	0
Rheumatoid arthritis Marked activity	Unabsorbed	32	2048	64
	Beef erythrocytes	16	2048	128
	Guinea pig kidney	4	2048	512

[*] Normal sheep erythrocytes.
[†] Sheep erythrocytes sensitied with specific amboceptor.
[‡] Reciprocal of highest dilution giving 2+ agglutination of erythrocytes.

rheumatoid arthritis had differential titers of 16 or more, while in the group of cases in Northern California high differential titers were associated mainly with severe rheumatoid disease and did not occur in many others with obvious activity. Since Rose's method was adhered to in all details these differences in results might represent variations in patients rather than differences in technique. Perhaps the rheumatoid arthritis observed in Northern California is a somewhat milder disease than that on the East Coast. Some cases in the present series, included under the diagnosis of rheumatoid arthritis, despite a typical clinical picture, may actually have had some other disease entity. On the other hand it must be pointed out that on the whole the sensitivity of the test, as carried out in this laboratory, was somewhat less than that indicated in Rose's paper, with both absolute and differential titers at lower levels. It is not clear at this time what technical feature might be responsible for these reported differences.

Rose *et al.* reported that rheumatoid arthritis was the only one of the disorders, sometimes attributed to "hypersensitivity," which produced elevated differential agglutination titers while rheumatic fever and visceral angiitis failed to do so. Our results agree in this respect. One case of ankylosing spondylitis with a high differential titer had, in contrast to Rose's observation, no peripheral joint involvement.

In the group studied the differential sheep cell agglutination test was of value in following the course of patients who initially had high differential titers. Titers observed over a period of 3-5 months in most cases reflected the clinical status. In patients with unequivocal improvement, the differential titer fell long before any change in the erythrocyte sedimentation rate. The test might thus be helpful as an objective aid for the evaluation of the results of treatment.

Summary. Results obtained with the differential sheep cell agglutination test of Rose *et al.* in a series of 160 patients are reported. High differential titers were observed almost solely in sera of patients with severe rheumatoid arthritis. In many others, with milder yet active disease, no significant titers were obtained. Adsorption with boiled guinea pig kidney frequently increased the differential titer of sera from active cases of rheumatoid arthritis. In patients with initial differential titers of 16 or more, tested over 3-5 months, the titers paralleled the clinical course.

TABLE II.
Differential Sheep Cell Agglutination Titer in Relation to Clinical Status of Patients Observed During a Period of Time.

Patient	Date	Clinical impression	Therapy	Differential aggl. titer
H	8/24/48	R.A.,* marked activity	Gold	128
	11/ 9	" " "	"	128
	12/21	" slight improvement	"	128
	2/ 1/49	" moderate activity	"	64
C	9/21/48	R.A., marked activity	"	16
	12/21	" " "	"	16
	1/25/49	" moderate activity	"	8
M	9/21/48	R.A., " "	"	16
	12/21	" " "	"	16
	1/25/49	" " "	"	16
L	8/10/48	R.A., " "	"	32
	12/21	" greatly improved	"	4
	1/25/49	" no activity	"	4
Ma	9/21/48	R.A., marked activity	Rest	32
	12/21	" minimal activity	"	4
B	9/21/48	R.A., marked activity	Gold	64
	12/21	" greatly improved	"	8
	1/25/49	" questionable activity continued improvement	"	2

* Rheumatoid arthritis.

these few patients tested and over a short period of time the differential titer did reflect the course of the disease (Table II).

Rose[2] demonstrated by electrophoretic analysis that the substance responsible for agglutination of sensitized sheep cells was a globulin in the beta-gamma fraction of serum. The heterophile antibody present in normal serum is adsorbed by guinea pig kidney. In serum from infectious mononucleosis the heterophile antibody can be removed by adsorption with boiled beef erythrocytes. Table III shows the agglutination of normal and sensitized sheep erythrocytes by adsorbed sera. The normal heterophile antibody of sera from active rheumatoid arthritis was adsorbed by guinea pig kidney. However, neither boiled beef erythrocytes nor guinea pig kidney removed the substance responsible for agglutination of sensitized sheep cells, thus differentiating it from the heterophile antibodies present in sera from normal individuals, from patients with infectious mononucleosis, or from persons who have been injected with horse serum. Thus the differential titer of sera from patients with active rheumatoid arthritis could frequently be increased by adsorbing with boiled guinea pig kidney, prior to the test.

The stability of the substance responsible for agglutination of sensitized sheep erythrocytes was investigated. With sterile sera kept at 4°C both absolute and differential agglutination titers remained essentially stable for 4-20 days. Minimal bacterial contamination quickly destroyed the activity. When stored at —70°C in the dry ice chest in sealed ampoules individual or pooled sera maintained their titer for at least 3 months.

Discussion. The results obtained confirm the claims of Rose *et al.*[2] that there is a substance in the serum of some patients with rheumatoid arthritis which agglutinates sensitized sheep erythrocytes. Adsorption of such sera with boiled guinea pig kidney or beef cells emphasizes the difference between this substance and other components of the serum which agglutinate sheep cells.

There are, however, important differences between the results reported by Rose *et al.* and those observed in the present series. In the New York series all patients with active

TABLE III.
Differential Sheep Cell Agglutination Test Applied to Sera Absorbed with Boiled Beef Erythrocytes and Boiled Guinea Pig Kidney.

Serum	Absorbed with	NS*	SS†	Differential aggl. titer
Normal human	Unabsorbed	8‡	16	2
	Beef erythrocytes	16	16	0
	Guinea pig kidney	0	0	0
Infectious mononucleosis	Unabsorbed	4096	4096	0
	Beef erythrocytes	64	128	2
	Guinea pig kidney	4096	4096	0
Rheumatoid arthritis Marked activity	Unabsorbed	32	2048	64
	Beef erythrocytes	16	2048	128
	Guinea pig kidney	4	2048	512

* Normal sheep erythrocytes.
† Sheep erythrocytes sensitied with specific amboceptor.
‡ Reciprocal of highest dilution giving 2+ agglutination of erythrocytes.

rheumatoid arthritis had differential titers of 16 or more, while in the group of cases in Northern California high differential titers were associated mainly with severe rheumatoid disease and did not occur in many others with obvious activity. Since Rose's method was adhered to in all details these differences in results might represent variations in patients rather than differences in technique. Perhaps the rheumatoid arthritis observed in Northern California is a somewhat milder disease than that on the East Coast. Some cases in the present series, included under the diagnosis of rheumatoid arthritis, despite a typical clinical picture, may actually have had some other disease entity. On the other hand it must be pointed out that on the whole the sensitivity of the test, as carried out in this laboratory, was somewhat less than that indicated in Rose's paper, with both absolute and differential titers at lower levels. It is not clear at this time what technical feature might be responsible for these reported differences.

Rose *et al.* reported that rheumatoid arthritis was the only one of the disorders, sometimes attributed to "hypersensitivity," which produced elevated differential agglutination titers while rheumatic fever and visceral angiitis failed to do so. Our results agree in this respect. One case of ankylosing spondylitis with a high differential titer had, in contrast to Rose's observation, no peripheral joint involvement.

In the group studied the differential sheep cell agglutination test was of value in following the course of patients who initially had high differential titers. Titers observed over a period of 3-5 months in most cases reflected the clinical status. In patients with unequivocal improvement, the differential titer fell long before any change in the erythrocyte sedimentation rate. The test might thus be helpful as an objective aid for the evaluation of the results of treatment.

Summary. Results obtained with the differential sheep cell agglutination test of Rose *et al.* in a series of 160 patients are reported. High differential titers were observed almost solely in sera of patients with severe rheumatoid arthritis. In many others, with milder yet active disease, no significant titers were obtained. Adsorption with boiled guinea pig kidney frequently increased the differential titer of sera from active cases of rheumatoid arthritis. In patients with initial differential titers of 16 or more, tested over 3-5 months, the titers paralleled the clinical course.

17024

Application of Filter Paper Partition Chromatography to Qualitative Analysis of Volatile and Non-Volatile Organic Acids.*

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In attempting to apply filter paper chromatography to organic acids one encounters streaking of many of the acids at the concentrations required for detection by color reaction, spreading or evaporation from the paper of the volatile members of the group, and too rapid migration, essentially with the solvent boundary, of the compounds with low solubility in water. Lugg and Overell[1] have reported a procedure which minimizes streaking and permits separation of many of the non-volatile organic acids. In an attempt to make filter paper chromatography more generally applicable to this class of compounds, a number of salts and derivatives were investigated. Of those tested, the potassium hydroxamate derivative appears most nearly to satisfy the requirements of the procedure. By its use it has been possible to separate most of the common organic acids with chain lengths of about 8 carbon atoms or less.

Methods. The potassium hydroxamates were synthesized from the methyl esters of the organic acids, essentially according to the procedure of Hauser and Renfrow[2]. Separate solutions of hydroxylamine hydrochloride (2.4 g or 0.033 mole in 15 ml methyl alcohol) and potassium hydroxide (2.8 g or 0.05 mole in 10 ml methyl alcohol) were prepared at the boiling point of methyl alcohol. Both were allowed to cool to 30-40°C, the one containing alkali was added with shaking to the hydroxylamine solution, and the mixture was placed in an ice bath for 5 minutes to permit complete precipitation of potassium chloride. To this mixture was added with thorough shaking, 0.017 mole of the methyl ester of the organic acid (or one-half that quantity of the methyl ester of a dicarboxylic acid). The mixture was filtered immediately with suction, and the precipitate rinsed with a few ml of methyl alcohol. The precipitate remaining in the funnel was discarded. Methyl alcohol was added to the filtrate to make a final volume of 30 ml if 0.017 mole of the ester was added. When 0.0085 mole was used, the hydroxamate was concentrated on the steam bath to a final volume of 15 ml, and for some of the hydroxamate derivatives, especially of the dicarboxylic acids, it was necessary to evaporate off most of the methyl alcohol and add water to obtain solution of the precipitate which formed. No determinations were made of the yields of the hydroxamates in their synthesis from the acid.

Details of the apparatus used and the technique for partition chromatography have been described.[3] For the chromatograms, 5 microliters (3×10^{-6} mole) of the potassium hydroxamate solution were used. A saturated solution of ferric chloride in n-butyl alcohol saturated with water was used as the color developing reagent.

Results. A number of solvents were found to be satisfactory for separating short-chain monocarboxylic acids, and R_F values for a number of such acids are given in Table I. Acids containing more than one carboxyl group frequently streaked, or gave numerous spots, or did not move sufficiently far from the initial position to permit a good separation in many of the solvents which were tried.

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* This document is based on work performed under contract W-7401-eng-49 for the Atomic Energy Project at the University of Rochester, and declassified in 1947 as MDDC 1485.

† Present address of authors: Birmingham Veterans Administration Hospital, Van Nuys, Calif., and the University of California, Los Angeles, Calif.

[1] Lugg, J. W. H., and Overell, B. T., *Nature*, 1947, **160**, 87.

[2] Hauser, C. R., and Renfrow, W. B., Jr., *Organic Syntheses*, 1939, **19**, 15.

[3] Dent, C. E., *Biochem. J.*, 1948, **43**, 169.

TABLE I.
R_F Values of Hydroxamate Derivatives of Organic Acids in Various Solvents on Whatman No. 1 Filter Paper.

	Solvent						
Hydroxamate	n-Hexyl alcohol	n-Amyl alcohol	n-Butyl alcohol	sec-Butyl alcohol	Methyl ethyl ketone	Isobutyric acid	Phenol
Formic	.06	.12	.40	.54	.22	.45	.57
Acetic	.23	.35	.51	.63	.40	.57	.70
Propionic	.43	.56	.68	.78	.61	.68	.78
Butyric	.63	.71	.79	.87	.75	.74	.80
Valeric	.73	.78	.86	.90	.84	.83	.84
Caprylic	.86	.88	.90	.91	.91	.87	.90
Pelargonic	.84	.85	.91	.90	.91	.88	.90
Capric	.89	.89	.92	.90	.90	.92	.95
Benzoic	.69	.73	.82	.86	.83	.79	.85
Phenylacetic	.73	.76	.83	.83	.84	.75	.86
Lactic	.14	.23	.42	.53	.28	.50	.66

TABLE II.
R_F Values of Hydroxamate Derivatives of Organic Acids in Phenol and in Isobutyric Acid on Whatman No. 1 Filter Paper.

	Solvent	
Hydroxamate	Phenol	Isobutyric acid
Oxalic	.14, .40	.23, .28, .32
Malonic	.11, .23	.19, .32
Succinic	.40, .72 orange	.45, .52 orange
Glutaric	.47	.37, .52
Adipic	.54, .67	.44, .60
Pimelic	.60, .73	.52, .69
Azelaic	.63, .74	.66 streaked
Sebacic	.89	.74, .89
Citric	.09, .23	.20, .29
Tartaric	.10	.19
Pyruvic	.59, .86	.54, .62 orange, .73

The most satisfactory solvents of those tested for these acids were isobutyric acid and phenol. R_F values for a number of these acids and for pyruvic acid are given in Table II, and a diagram of a two dimensional chromatogram is given in Fig. 1.

Discussion. Converting the organic acids to their hydroxamate derivatives increases the polarity, decreases the volatility, and makes possible the use of a convenient and reasonably sensitive color reaction for development of the chromatogram without increasing the molecular weight to such a degree that solubility differences between members of a homologous series are seriously diminished. In general, the color reaction is sufficiently sensitive to detect on the finished chromatogram a spot containing in the order of 10^{-7} mole of acid, assuming a quantitative conversion of the ester to the hydroxamate in the preparative procedure, while use of a mixture containing more than about a milligram of any one component is likely to lead to spreading or streaking due to the overloading of the paper. With proper controls, a rough quantitative estimation may be made of the amount of acid in a spot by judging from the size of the spot and the intensity of the color.

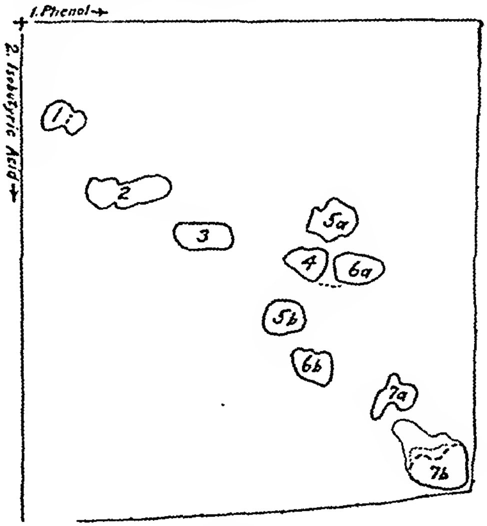

FIG. 1.

Diagram of a two dimensional chromatogram of hydroxamate derivatives of organic acids. The hydroxamates were applied at the point indicated by the cross. Phenol was used as the first solvent and isobutyric acid as the second. The numbers refer to the acids as follows:

1. tartaric
2. malonic
3. succinic
4. lactic
5. adipic
6. pimelic
7. sebacic

Disadvantages in the use of the hydroxamate derivatives include the manipulations involved in their preparation and the multiple spots obtained from the acids with multiple functional groups. The multiple spots are presumably due to failure to carry the preparative reactions to completion.

The colors in the developed chromatogram are relatively stable, although continued exposure to strong light causes a loss of contrast, principally by action on the ferric chloride background. Intense spots of the ferric hydroxamates tend to spread to other papers in contact with them, so that in filing it is well to insert blank sheets of paper between the chromatograms.

One dimensional chromatogram may be made on filter paper previously impregnated with ferric chloride, in which case the chromatogram may be examined visually at any stage of the development, but better results are usually obtained by use of non-impregnated paper.

Summary. A procedure for the application of filter paper partition chromatography to the separation of both volatile and non-volatile organic acids with chain lengths of about eight carbons or less is described. The potassium hydroxamate derivatives of the organic acids are prepared by reacting the methyl ester with about a two-fold excess of a mixture of potassium hydroxide and hydroxylamine in methyl alcohol. The hydroxamate derivatives obtained from about 10^{-6} mole of each of the esters are applied to the filter paper, the chromatogram developed with suitable solvents, and then sprayed with ferric chloride to make the derivatives visible as purple spots on a yellow background. Isobutyric acid and phenol gave the best results of the solvents tried for two-dimensional chromatograms of the dicarboxylic acids. R_F values for a number of hydroxamate derivatives are given for several solvents.

17025

Destructive Action of Human Cancer Extracts on Red Blood Cells *in vitro*.*

LUDWIK GROSS.

From the Cancer Research Unit, Veterans Administration Hospital, Bronx, N. Y.

It was recently observed in this laboratory that centrifugated or filtered extracts prepared from spontaneous mouse mammary carcinomas hemolyze mouse erythrocytes *in vitro.*[1]

Experiments reported in this study were designed with the purpose of determining whether extracts prepared from human cancer also exert a destructive action on red blood cells *in vitro*.

Materials and methods. Tumor extracts. Sterile specimens of tumors were obtained from the operating room.† The tumor tissue was weighed, cut with scissors into small pieces, then ground thoroughly for several minutes in a porcelain mortar, 0.85% solution of sodium chloride being added to obtain cell suspensions varying in concentration from 20 to 30%. The suspensions thus obtained were cleared from cells by 2 successive centrifugations at 5,000 r.p.m. for 10 minutes each; the final supernatant fluid was then used, and designated "tumor extract." In several cases, tumors of hard, fibrous consistency had to be ground into fine cell suspensions in a Latapie Grinding Apparatus (Thomas), and then centrifugated.

Cell suspensions prepared from human

---

* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

[1] Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 292.

† Most of the tumor specimens were obtained from the operating room of our hospital (Veterans Administration Hospital, Bronx, N. Y.); 10 of the 12 specimens of human breast carcinomas were obtained from the Memorial Hospital, New York City.

TABLE I.
Action of Cell-Free Human Tumor Extracts on Human RBC *in vitro.*

Tumor specimen				Results of the RBC test *in vitro*	
No. of test	Specimen diagnosis	Blood group of patient	RBC used for test	Hemolysis after 48 hr incub. 37°C	Agglutination after 24 hr incub. 37°C
1.	Carcinoma, breast (F)	O pos.	O pos.	+	0
2.	” ” (F)	O pos.	O pos.	+	0
3.	” ” (F)	O pos.	O pos.	+	sl[†]
4.	” ” (F)	B pos.	B pos.	+	+
5.	” ” (M)	A pos.	A pos.	+	0
6.	” ” (F)	O pos.	O pos.	sl[†]	sl[†]
7.	” ” (F)	O pos.	O pos.	0[‡]	+
8.	” ” (F)	O neg.	O neg.	sl[†]	0
9.	” ” (F)	ND[*]	O pos.	+	sl[†]
10.	” ” (F)	ND[*]	O pos.	+	0
11.	” ” (F)	ND[*]	O pos.	+	+
12.	” ” (F)	ND[*]	A pos.	+	+
			B pos.	+	+
13.	Carcinoma, stomach (M)	A pos.	A pos.	0	+
14.	” ” (M)	O neg.	O neg.	+	sl[†]
15.	” ” (M)	A pos.	A pos.	+	+
16.	” ” (M)	A pos.	A pos.	+	sl[†]
17.	” ” (M)	B pos.	B pos.	+	0
18.	” rectum (M)	B pos.	B pos.	sl[†]	0
19.	” antrum (M)	O neg.	O neg.	0[‡]	sl[†]
20.	” ” (M)	A neg.	A neg.	+	0
21.	” metastatic (primary undetermined) (M)	A pos.	A pos.	sl[†]	sl[†]

[*] ND—Blood group not determined.
[†] sl Slight.
[‡] Minimal hemolysis noticed.
(F) female.
(M) male.

breast cancer formed a fatty layer on the surface of the supernatant fluid following centrifugation; this fat had to be separated from the extract by passing the supernatant fluid through a sterile voile filter.

In a few instances, apparently infected tumors such as carcinoma recti, or that of the antrum, were used for the tests. To assure bacterial sterility, extracts prepared from such tumors were passed through a Seitz filter, and the filtrate was used for the tests. In addition, filtrates, as well as centrifugated extracts, were prepared also from several other bacteriologically sterile tumors.

The entire procedure followed in the preparation of the extracts was aseptic, and the extrates, or filtrates, used for the tests were found to be bacteriologically sterile, as evidenced by negative inoculations of ordinary culture media.

Normal Tissue Extracts. Control experiments were performed with cell-free extracts prepared from various freshly obtained and sterile human tissues: specimens of healthy muscle were obtained from patients in whom a limb was amputated; specimens of thyroid gland were obtained from patients in whom thyroidectomy was performed because of thyreotoxicosis; specimens of hypertrophic, but otherwise normal, breast gland were obtained from 5 males operated on for gynecomastia, and from one female in whom a plastic breast operation was performed; a specimen of placenta was obtained from a young woman in whom Caesarean Section was performed; finally, 2 small specimens of apparently normal liver were obtained from patients in whom a diagnostic liver biopsy was performed. From these various tissue specimens, centrifugated, cell-free, and bacteriologically sterile extracts were prepared in a manner identical with that described for the preparation of the tumor extracts.

Red Blood Cells (RBC). Freshly drawn, oxalated red blood cells from healthy human donors, as well as in some instances also from

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The colors in the developed chromatogram are relatively stable, although continued exposure to strong light causes a loss of contrast, principally by action on the ferric chloride background. Intense spots of the ferric hydroxamates tend to spread to other papers in contact with them, so that in filing it is well to insert blank sheets of paper between the chromatograms.

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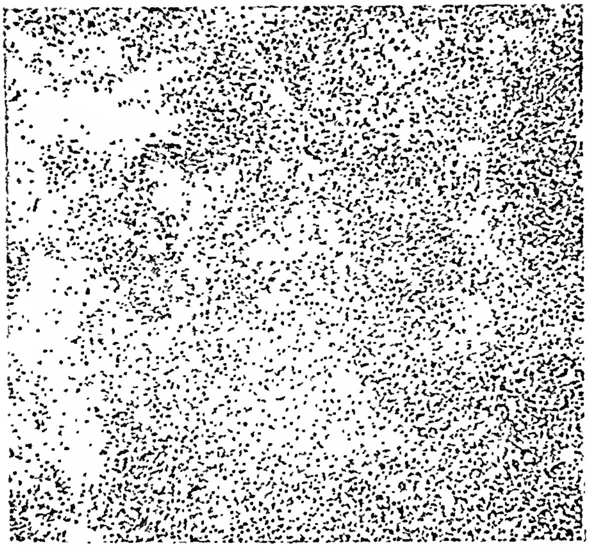

FIG. 2.
A slight clumping action of an extract from a human stomach carcinoma on human erythrocytes after 18 hours of incubation at 37°C. Mag. ×67.

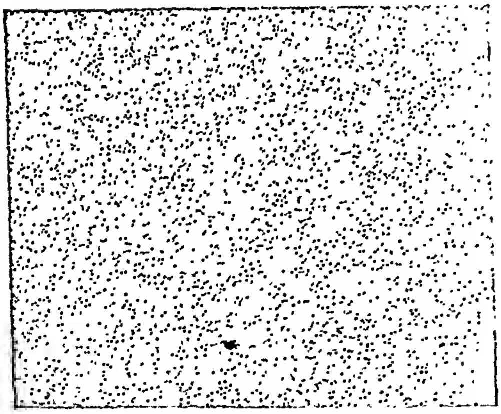

FIG. 3.
Human erythrocytes, 1% suspension, after 24 hours incubation at 37°C. Control slide. Mag. ×67.

tracts on Human RBC in Vitro. Most of the extracts prepared from human tumors were found to hemolyze (Fig. 4) human erythrocytes *in vitro* after an incubation for 48 hours at 37°C (Table I). Control tubes containing human red blood cell suspensions in physiological saline solution, and incubated simultaneously under otherwise identical conditions, remained unaltered.

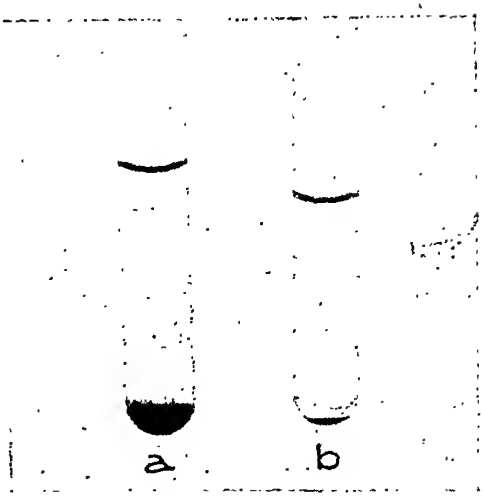

FIG. 4.
a. "Slight" hemolysis resulting from exposure (48 hours at 37°C) of human erythrocytes to a filtered extract prepared from human breast carcinoma. b. Control tube containing erythrocytes suspended in physiol. saline solution; no hemolysis.

In 8 experiments the tumor extracts were passed through Seitz filters, and the filtrates, as well as the corresponding centrifugated extracts, were tested for their hemolytic potency. In 3 instances the tumor extracts lost their guinea pigs, rabbits, chickens, and mice, were washed twice at 2,500 r.p.m. for 3 minutes each, and final one per cent suspensions of the red blood cells in 0.85% solution of sodium chloride were prepared. The washed red blood cell suspensions were freshly prepared for each test. In all instances, except when indicated otherwise in Table I, human erythrocytes of the same type were used as that of the patient from whom the specimen had been removed.

Technic of the Test. The test was performed in the following manner: one cc of the 1% RBC suspensions was mixed gently in a small (10 x 75 mm) tube with doses of the tumor, or normal tissue, extracts varying from 1 to 0.25 cc, and placed in an incubator at 37°C for 48 hours. The presence or absence of agglutination was determined after 24 hours of incubation at 37°C; agglutination, if any present, was graded "slight," "1+," and "2+." The presence or absence of hemolysis was determined by inspecting the tube, without previous centrifugation, after incubation at 37°C for 48 hours; hemolysis present only over the sediment was graded "slight"; if hemolysis was stronger so as to diffuse in the supernatant fluid to a level extending at least for 1 cm above the sediment, it was graded "1+."

Experimental. "Agglutinating" Action of the Human Tumor Extracts on RBC in Vitro. The curious clumping action of tumor extracts on red blood cells, observed in experiments with transplanted mouse carcinomas,[2] was reproduced in some of the tests performed with human cancer extracts mixed with human erythrocytes. The clumping potency of the tumor extracts was not a constant phenomenon (Table I). In some instances, it occurred promptly; in others, only very slightly, and with a considerable delay, or it was missing entirely. Even in the more pronounced cases, however, the examination of the sediment under the microscope failed to show a typical picture of agglutination (Fig. 1 to 3). The clumping of the cells was usually only slight, if at all present, and was far from being uniform; only some of the cells were affected; accumulation of either debris, or some unidentified precipitated masses could also be noticed. The interpretation of these findings was not facilitated by the observation that frequently a slight precipitation occurred also in control tubes containing tumor filtrates only, without erythrocytes, and incubated at 37°C for 24 hours.

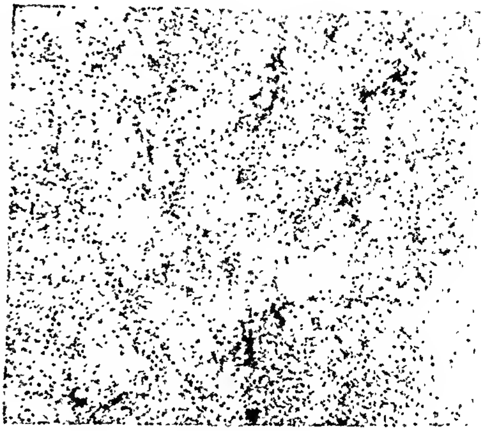

FIG. 1.

Agglutinating action of human breast carcinoma extract on human erythrocytes *in vitro* after 24 hours of incubation at 37°C. Mag. ×67.

In 8 experiments the tumor extracts were passed through a Seitz filter and the filtrates were mixed with the erythrocytes; in all these cases the agglutinating potency of the extracts was not diminished by filtration. In fact, the agglutinating potency of the filtrates appeared to be somewhat more distinct than that of the centrifugated extracts.

In 10 experiments the tumor extracts were heated to 56°C for 30 minutes in a water bath prior to mixing them with red blood cells. All such preheated extracts were found to have lost their ability to agglutinate erythrocytes *in vitro*.

A series of experiments was performed in which the human tumor extracts were mixed with erythrocytes of mice, rats, guinea pigs or chickens. In all these instances in which such extracts were found to agglutinate human red blood cells, they were also found to exert a similar agglutinating action on the erythrocytes of the various different species of the animals tested.

Hemolytic Action of Human Tumor Ex-

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[2] Gross, L., *J. Immunol.*, 1948, 59, 173.

TABLE II.
Action of Cell-Free Extracts from Normal Human Tissues on Human RBC *in vitro.*

Organs tested	Blood group of patient	No. of individual extr. tested	Blood group or RBC used	No. of extr. causing hemolysis	No. of extr. causing agglut.
Muscle	A pos.	4	A pos.	0	1*
	A neg.	1	A neg.	0	0
,,	O pos.	2	O pos.	0	0
	O neg.	1	O neg.	0	0
Placenta	B pos.	1	B pos.	0†	0
Thyroid	B pos.	1	B pos.	0	0
,,	O pos.	1	O pos.	0†	0
,,	O pos.	1	O pos.	0	0
,,	AB pos.	1	AB pos.	0	0
Breast (F)	A pos.	1	A pos.	0	0
,, (M)	O pos.	4	O pos.	1*	0
Liver	A neg.	1	A neg.	0†	0
	B pos.	1	B pos.	0	0

Each extract was prepared from a different patient. The agglutination was read after 24 hours of incubation at 37°C. The hemolysis was read after 48 hours of incubation at 37°C.

* "Doubtful."

† Slight hemolysis after 72 hours of incubation at 37°C.

had been removed; these mixtures were then incubated, at 37°C, for 48 hours (Fig. 5). There was only one instance of a "questionable" agglutination (Table II.); all other sediments were negative for clumping when inspected after either 24 or 48 hours of incubation. Of the 21 individual extracts tested, only one produced a minimal hemolysis after 48 hours of incubation; in 3 additional instances, a slight hemolysis resulted after 72 hours of incubation at 37°C.

Discussion. In previous experiments,[1,2] mouse mammary carcinoma extracts were found to hemolyze mouse erythrocytes, *in vitro,* after 2 to 3 hours of incubation at 37°C. Agglutination of the red blood cells by mouse tumor extracts was found to be less frequent, unless transplanted tumors were used for the preparation of the extracts.[2] Both observations have been recently confirmed by Zimmerman[3] and Salaman.[4]

At first, similar experiments performed with human tumor extracts in this laboratory gave negative results, except for occasional agglutination[5] (Fig. 6); in these preliminary tests the readings were made after a brief incubation at 37°C, not exceeding 3 or 6 hours. In the case of experiments with mouse tumor extracts a longer incubation was not necessary, and for that reason a similar procedure was followed in these tests dealing with human cancer. Later on, however, the human tumor extracts were allowed to act on human erythrocytes for longer periods of time; this modification in the technic of the test lead to positive results: agglutination, and hemolysis were found to result frequently. Even after a prolonged incubation, however, the hemolytic action of the human tumor extracts on human erythrocytes was found to be somewhat less pronounced than the similar action of mouse carcinoma extracts on mouse red blood cells.

The hemolytic, and particularly the agglutinating actions of the extracts prepared from various human tumors were far from constant; in some instances the hemolysis and/or agglutination occurred promptly and to a pronounced degree. In other cases, the destructive action of the tumor extracts was slight, and delayed. Since, however, the various extracts were prepared from different tumors, it was reasonable to expect differences in their ability to destroy the erythrocytes. Similar differences in the potency of the individual tumors were also observed in experiments dealing with mouse carcinomas.[2]

The question immediately arose whether cell-free extracts prepared from normal human tissues would also exert a similar destructive hemolytic potency after filtration; in 4 instances, the hemolytic potency of the extract was substantially diminished, but detectable, after filtration. Finally, in one instance, the hemolytic potency of the extract was not diminished after filtration.

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[3] Zimmerman, H. M., (Montefiore Hospital, Bronx, N. Y.), personal communication to the author.

[4] Salaman, M. H., *Brit. J. Cancer,* 1948, **2**, 253.

[5] Gross, L., unpublished experiments.

In 10 successive experiments, human tumor extracts were heated to 56°C for 30 minutes in a water bath, prior to mixing them with the red blood cells. In all instances the hemolytic potency of these extracts was destroyed by heating.

In 4 experiments, centrifugated tumor extracts, separated from the tumor cells, were incubated at 37°C for 4 hours, and then mixed with the erythrocytes. The potency of these pre-incubated extracts did not appear to be diminished, as compared with the corresponding control samples of fresh tumor extracts. This was in marked contrast to similar experiments showing that mouse tumor extracts lose their hemolytic potency when separated from the tumor cells, and incubated at 37°C for a few hours.[2]

An attempt was made to determine whether human tumor extracts would also hemolyze red blood cells of other species of animals. Experiments dealing with this question encountered, however, a rather unexpected difficulty; thus, it was found that washed mouse erythrocytes undergo spontaneous hemolysis when suspended in physiological saline solution, and incubated at 37°C for 12 to 24 hours; erythrocytes of rabbits, and to a lesser degree those of guinea pigs, were also found to be not suitable for a long incubation at 37°C.

In a few cases only, using potent human tumor extracts, was it possible to observe a slight hemolytic action of the human tumor extracts on the red blood cells of mice, rabbits, and guinea pigs, occurring comparatively promptly, and at a time when the control tubes with the respective erythrocytes suspended in physiological saline solution did not yet show any trace of hemolysis. The washed chicken erythrocytes appeared to be more resistant to spontaneous hemolysis when incubated at 37°C, and for that reason were found to be better suitable for these tests than the red blood cells of guinea pigs, rabbits or mice. In a few instances it was found that human tumor extracts slightly hemolyzed chicken erythrocytes after incubation for 48 hours at 37°C.

Experiments with Extracts Prepared from Non-Malignant Tumors. Four experiments were made with extracts prepared from non-malignant human tumors, *i.e.* from 3 prostatic adenomas (one centrifugated and 2 filtered extracts), and from a uterine myoma. In all instances washed erythrocytes of the same type were used for the tests as the type of the patient from whom the specimen had been removed. The results were as follows: the 2 filtrates prepared from the prostatic adenomas agglutinated the red blood cells after 24 hours of incubation at 37°C; the other 2 extracts did not agglutinate the erythrocytes. All 4 tests were negative for hemolysis after 48 hours of incubation at 37°C; after additional 24 hours of incubation, however, the prostatic adenoma extract, one of the 2 prostatic filtrates and the uterine myoma extract, produced a slight hemolysis of the corresponding erythrocytes.

Action of Normal Human Tissue Extracts on Human RBC in Vitro. Sterile, cell-free, centrifugated extracts freshly prepared from various normal human tissues, were mixed with 1% suspensions of washed human erythrocytes of the same type as that of the patient from whom the respective specimen

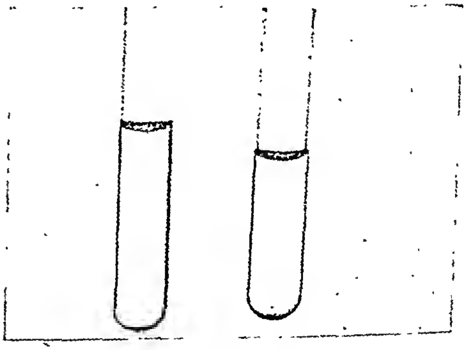

FIG. 5.

Exposure of human erythrocytes (48 hours at 37°C) to the action of an extract prepared from human breast gland (gynecomastia). a) Extract from breast gland (1 cc), and RBC. No hemolysis. b) Physiol. saline solution, and RBC. Control tube. No hemolysis.

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Cell Proliferation Accelerating and Inhibiting Substances in Blood Serum During Pregnancy.*

EARL R. NORRIS AND JOHN J. MAJNARICH.

From the Department of Biochemistry, University of Washington, Seattle.

In blood serum there are two types of factors which affect cell proliferation in bone marrow cell and tissue cell cultures *in vitro*.[1] Normal blood serum will accelerate the rate of cell proliferation in bone marrow cultures because of a predominance of accelerating over inhibiting factors. Serum from cases of neoplastic disease, pernicious anemia, aplastic anemia and leukemia have a ratio of factors such that a supplement of the serum will inhibit normal cell proliferation. In a series of conditions previously reported,[2] the sera, from 7 cases of pregnancy, inhibited the rate of cell proliferation.

A further study was made of the effect of blood serum from cases of pregnancy on cell proliferation in bone marrow cultures *in vitro*. The technique used for the culturing of bone marrow cells was the same as that previously described.[2] Rabbit bone marrow was used in the experiments reported in this paper.

Fig. 1 gives the response of the cells of bone marrow cultures *in vitro* to the addition of human pregnancy blood serum at various stages of pregnancy. Circles connected by a line represent cases in which three or more specimens of blood serum were obtained at the intervals indicated. Determinations have been made on thirty cases in addition to those shown in Fig. 1, especially in the latter stages of pregnancy. The results all fall close to the order of magnitude of values given in the curves shown and are omitted to prevent overcrowding of the graph. During pregnancy there was an increase in factors which inhibit normal cell proliferation, with a tendency toward an increasing inhibition with the progress of pregnancy. In the later stages of pregnancy, the blood sera were strongly inhibitory of normal cell proliferation similar to the blood sera from cases of neoplastic disease, pernicious and aplastic anemia, and leukemia. The anemia associated with pregnancy is consistent with the change in normal cell accelerating and inhibiting factors as is also the anemia associated with neoplastic disease.

Fig. 2 gives the response of bone marrow cultures *in vitro* to the blood serum of pregnant rats at various stages of pregnancy. The blood serum showed a progressive increase in inhibiting factors up to the time of parturition. The amniotic fluid was obtained from the uterus of the pregnant rats on approximately the eleventh and fifteenth day of pregnancy and at the time of birth. The amniotic fluid becomes strongly inhibitory during the pregnancy, but at the time of birth there was a considerable excess of normal cell, proliferation accelerating factors in the fluid.

At various stages of pregnancy, the rats were sacrificed and the cells of the embryos or fetuses cultured by a technique similar to that used for culturing normal tissue cells and cancer cells *in vitro*.[3,4] The entire embryos were disintegrated in a modified Waring blender as previously described for the preparation of tissue cell suspensions with the addition of Tyrode's solution without glucose. The cell suspension was incubated in 5 ml rubber capped vials at 37°C. Ten mg of acid hydrolyzed casein per ml of cell suspension was added as a neutralized solution of commercial casein hydrolysate. One half mg of

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Norris, E. R., and Majnarich, J. J., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 229.

[2] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 483.

[3] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 488.

[4] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 492.

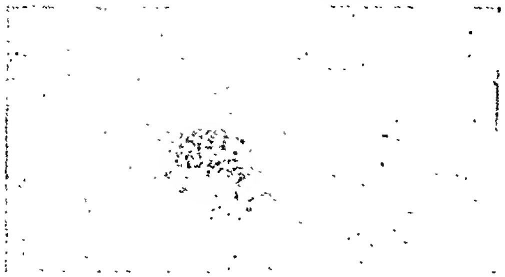

FIG. 6.

Agglutination of human erythrocytes by a human carcinoma (stomach) extract after an incubation, in test tube, at 37°C, for 6 hours. A drop of the sediment was then placed on a slide. The agglutination is visible macroscopically. Mag. ×1½.

action on human erythrocytes; this point was of particular interest in view of the fact that preincubated extracts from certain mouse tissues,[6] as well as *slices*[7,8] of normal animal or human tissues, had been found to hemolyze red blood cells *in vitro* after a prolonged incubation at 37°C. Accordingly, a series of experiments was performed with freshly prepared cell-free extracts from various human organs. With insignificant exceptions, the results of these tests were negative.

Anemia is a frequent associate of cancer. Some of the tumors, such as carcinomas of the intestinal tube, may ulcerate and bleed, giving apparently sufficient reason for the resulting anemia. In other instances, however, no bleeding occurs, and yet anemia also eventually develops. The exact cause of the progressive anemia so frequently encountered also in non-bleeding, and not ulcerated cases of cancer, has been a matter of controversy.

Experiments reported in this paper suggest that certain malignant tumors in man may exert a direct destructive action on the red blood cells *in vitro*. Should a similar phenomenon occur in the living host, a logical explanation of at least one of the causes of anemia in certain cases of cancer would be at hand. Such an explanation would be consistent with reports of recovery from anemia of patients relieved from tumors by surgical procedures.[9] On the other hand it appears difficult to assume that all cases of anemia in cancer are caused by a direct destruction of the red blood cells in the hosts; frank hemolytic anemia has been occasionally observed in neoplastic diseases,[10,11] but is far from being either frequent or usual. It is apparent, therefore, that some other mechanism would have to be also, at least partially, responsible for the development of anemia in certain cases of cancer. Thus, theoretically at least, one could assume that the destructive action of a diffusible substance, either liberated or produced by the tumor, may not be limited to erythrocytes, but may affect, in certain instances, among some other cells, also those of the bone marrow. This, however, is speculation only at the present time.

The fact remains unchanged, nevertheless, that human cancer cells either liberate or secrete a thermolabile substance which, *in vitro* at least, exerts a slow acting, but destructive influence on human erythrocytes.

Summary. Experiments reported in this paper suggest that extracts prepared from human cancer occasionally agglutinate, and frequently hemolyze red blood cells *in vitro*. These phenomena, but particularly the hemolysis, become evident only after a prolonged incubation of the tumor extracts with the red blood cells at 37°C. The agglutination is evident after 24 hours of incubation or earlier; the hemolysis requires 48 hours of incubation. Heating of the tumor extracts for 30 minutes to 56°C, prior to mixing them with the red blood cells, destroys their ability to either agglutinate or hemolyze the erythrocytes.

Mrs. Ruth G. Zahler rendered very ably technical assistance in this study. Dr. B. S. Gordon, Chief of the Clinical Laboratory was responsible for the pathological diagnosis of the slides. Mr. S. Shapiro, Chief of Medical Illustration, was responsible for the photographs.

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[6] Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 341.

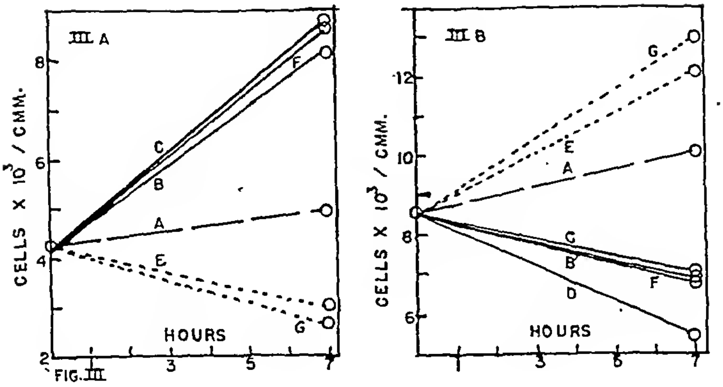
[7] Maegraith, B. G., Martin, N. H., and Findlay, G. M., *Brit. J. Exp. Path.*, 1943, **24**, 58.

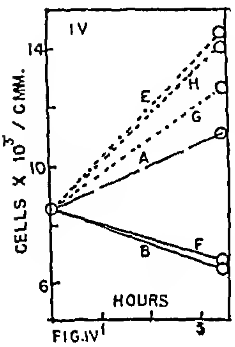
[8] Bruckmann, G., and Wertheimer, E., *Brit. J. Exp. Path.*, 1945, **26**, 217.

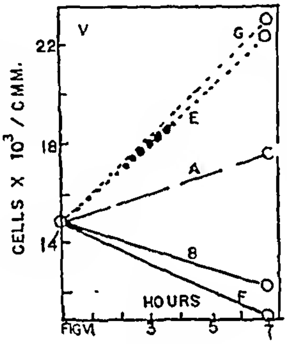
[9] Jones, E., and Tillman, C., *J.A.M.A.*, 1945, **128**, 1225.

[10] Wintrobe, M. M., *Clinical Hematology*, Philadelphia, Lea & Febiger, 1946.

[11] Stats, D., Rosenthal, N., Wasserman, L. R., *Amer. J. Clin. Path.*, 1947, **17**, 585.







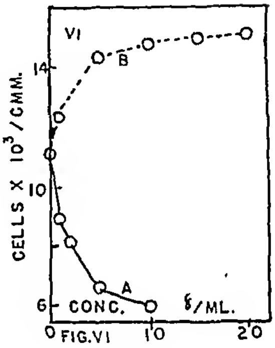


FIG. 3.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from the uterus of a non-pregnant rat (III A) and from the uterus and embryos of a obtained with 0.1 ml of blood serum from rats, at different stages of pregnancy, added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; C, 1×10^{-6} γ of Vitamin B_{14} per ml; D, 1×10^{-2} γ of Vitamin B_{14} per ml; E, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; F, 0.1 ml of normal human blood serum; G, 0.1 ml of blood serum from a person with cancer.

FIG. 4.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells, from rat fetuses of about 10 days. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; E, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; F, 0.1 ml of normal human blood serum; G, 0.1 ml of human cancer blood serum; H, 0.1 ml of human leukemia blood serum.

FIG. 5.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from rat fetuses of about 15 days. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ per ml of xanthopterin; E, 5 γ per ml of 2-amino-4-hydroxy-7-methyl pteridine; F, 0.1 ml of normal human blood serum; G, 0.1 ml of human cancer blood serum.

FIG. 6.

The effect of various concentrations of pteridines upon the rate of cell proliferation *in vitro* in a suspension of cells from rat fetuses of about 10 days. A, xanthopterin; B, 2-amino-4-hydroxy-7-methyl pteridine.

cells produced from the uterus of a non-pregnant rat. The non-pregnant uterus gave the response obtained with normal tissue cells *in vitro*. The response of the uterus containing the implanted embryos at an early stage gave the opposite response to that of normal cell proliferation. Xanthopterin, Vitamin B_{14}[5]

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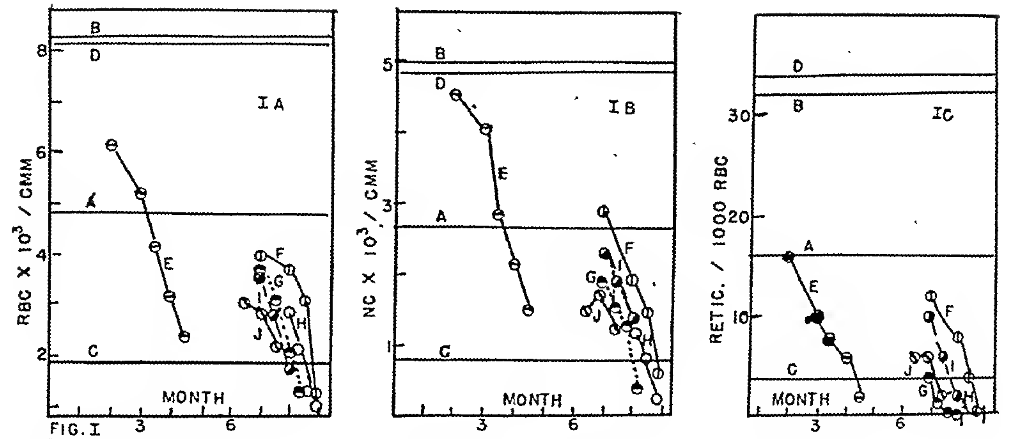
[5] Norris, E. R., and Majnarich, J. J., *Science*, in press.



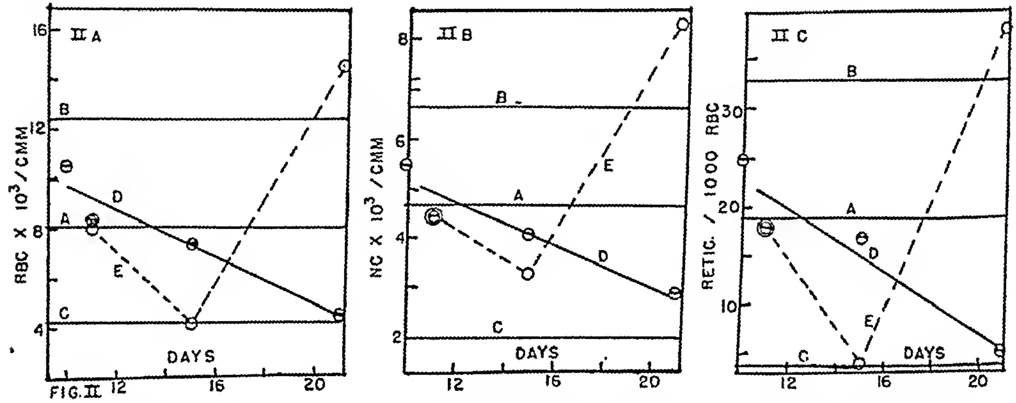


FIG. 1.

The effect of blood serum from cases of human pregnancy at different periods of pregnancy upon the rate of cell proliferation in bone marrow cell cultures *in vitro*. The initial concentration of cells in the bone marrow cell suspension used was red blood cells (RBC) 4840/cmm; nucleated cells (NC) 3240/cmm; reticulocytes 10/1000 RBC. Time of incubation of bone marrow cultures is 8 hours. A, indicates the level of response obtained without supplement; B, indicates the level of response obtained with 5 γ of xanthopterin per ml of suspension; C, indicates the level of response with 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml of suspension; D, the level of response with normal human serum; E, F, G, H, I, J, the response obtained with serum from cases of pregnancy at the period of pregnancy indicated. One tenth ml of serum was added to 2 ml of bone marrow suspension.

FIG. 2.

The effect of the blood serum and amniotic fluid of pregnant rats upon cell proliferation in bone marrow cultures *in vitro*, at various stages of pregnancy. A, is the level of response obtained with no supplement; B, is the level of response obtained with a supplement of 5 γ of xanthopterin per ml of bone marrow suspension; C, is the level of response obtained with 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml of bone marrow suspension; D, is the response obtained with 0.1 ml of blood serum from rats, at different stages of pregnancy, added to 2 ml of bone marrow suspension; E, is the response obtained with 0.1 ml of amniotic fluid from the uterus of pregnant rats, at different stages of pregnancy, added to 2 ml of bone marrow suspension.

tryptophan was added per ml of cell suspension, and supplements as indicated below.

In the earliest stages of pregnancy, it was not possible to separate the implanted embryos from the uterus so that the entire uterus containing the embryos was disintegrated and a cell suspension obtained. Fig. 3 gives response obtained with cells of the uterus from a rat in the early stages of pregnancy compared with that of a suspension of

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Spectrophotometric Method for Assay of Serum Antiprotease: Clinical Applications.*

H. H. TALLAN,† E. E. CLIFFTON,‡ AND G. R. DOWNIE.§
(Introduced by S. C. Harvey.)
From the Department of Surgery, Yale University School of Medicine.

Variation in the level of antiproteolytic activity in the serum has been studied in some detail since the presence of this factor was first reported in 1893.[1] The methods for determination of this activity in the past have largely been antifibrinolytic in nature,[2-5] and are not entirely satisfactory because of the subjectivity of the determinations and the variations in result due to differences in substrate and slight variations in handling of the tubes containing clots. A simpler and more objective procedure was described recently from this laboratory.[6] This was still time-consuming, and a more rapid, objective method of assay, to be described, was developed. The results obtained on examination of 250 sera by this method and by the previously reported clot formation method were collected and a statistical analysis was made of the correlation between the two tests.

Materials. 1. Trypsin‖ solution 70 mg% dissolved in veronal buffer, shaken well and filtered, kept cold until used and prepared daily.

2. Fibrinogen¶ solution, 0.5% in veronal buffer.

3. Trichloracetic acid (USP), 16% solution.

4. Sodium hydroxide (reagent grade), 1 N.

5. Saline solution, 0.9%.

6. Veronal buffer,[6] pH 7.4.

7. Folin-Ciocalteau phenol reagent,†† diluted 1:3 with distilled water.

Method. Blood is drawn into dry centrifuge tubes using a dry syringe and needle. Serum is obtained by centrifugation. The serum is diluted to 1:40 with saline, and 0.5 ml of the serum dilution is transferred to a 13 x 100 mm pyrex tube. Two similar tubes for uninhibited trypsin are set up with 0.5 ml of saline. A blank is prepared with 1.5 ml veronal buffer and is carried through the routine, except that trypsin is not added.

All tubes, with the blank tube first, are placed in an ice water bath. When temperature equilibrium has been reached (10 minutes), 1 cc of trypsin solution is added to each tube, the tubes are shaken, and returned to the bath at 30 second intervals (in order). After 30 minutes, in the same order and with the same time interval, 2 ml of fibrinogen are added to each tube. After the addition, each tube is shaken slightly and placed in a water bath at 37°C for 30 minutes.

At the end of this time 5.0 ml of trichlor-

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[1] Hildebrandt, H., *Virchows Arch. f. Path. Anat.*, 1893, **131**, 5.

[2] Brieger, L., and Trebing, J., *Berl. Klin. Woch.*, 1908, **45**, 1349.

[3] Jobling, J. W., and Peterson, W. F., *J. Exp. Med.*, 1913, **19**, 459.

[4] Jobling, J. W., Peterson, W. F., and Egstein, A. A., *J. Lab. and Clin. Med.*, 1915, **1**, 172.

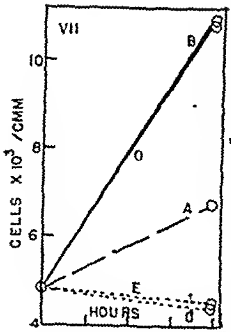
[5] Grob, D. (a) *J. Gen. Physiol.*, 1942, **26**, 405; (b) *J. Gen. Physiol.*, 1942, **26**, 423.

[6] Clark, D. G. C., Cliffton, E. E., and Newton, F. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 276.

‖ Armour trypsin powder.

¶ Armour bovine fibrinogen; Fraction I from bovine plasma.

†† Hartman-Leddon Company.



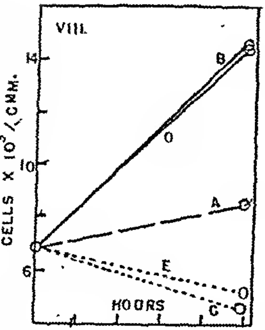


FIG. 7.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from rat fetuses taken at the time of birth. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; C, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; D, 0.1 ml of normal human blood serum; E, 0.1 ml of human cancer blood serum.

FIG. 8.

The effect of various supplements upon the rate of cell proliferation *in vitro* in a suspension of cells from rat young about 12 hours after birth. The following supplements were added to 2 ml of cell suspension: A, no supplement; B, 5 γ of xanthopterin per ml; C, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml; D, 0.1 ml of normal human blood serum; E, 0.1 ml of human cancer blood serum.

and normal blood serum accelerated the rate of cell proliferation of the non-pregnant uterus and inhibited proliferation of the cells of the pregnant uterus. Two-amino-4-hydroxy-7-methyl pteridine and cancer serum inhibited cell proliferation in the non-pregnant uterus cell suspension and accelerated the rate of cell proliferation in the pregnant uterus cell suspension.

Fig. 4, 5 and 6 give the response of cell suspensions obtained from fetuses on approximately the 10th and 15th day of pregnancy. The response was the same as that obtained with the uterus in the early stages of pregnancy described above and also the same as that obtained with neoplastic tissue cell suspensions.[4]

Fig. 7 and 8 give the response of cell suspensions obtained from the fetuses or young at the time of, and soon after birth. The response is that of normal cell suspensions. At some time between approximately the 15th day of pregnancy and the 21st day or the time of parturition, there was a complete change in the response of the cell proliferation to pteridines. In the early embryos and the fetuses up to at least the 15th day, the proliferation of the cells obtained as a suspension was inhibited by xanthopterin and accelerated by 2-amino-4-hydroxy-7-methyl pteridine. At the time of birth the rate of cell proliferation of the cells obtained from the young was accelerated by xanthopterin and inhibited by 2-amino-4-hydroxy-7-methyl pteridine.

Summary. 1. Two types of factors which affect cell proliferation have been observed in blood serum, one of which accelerates the rate of normal cell proliferation, and the other inhibits the proliferation of normal cells. Normal blood serum contains a predominance of factors which accelerate the rate of normal cell proliferation. During pregnancy there is a progressive change in the balance of factors which affect cell proliferation such that the factors which inhibit proliferation of normal cells become predominant.

2. The rate of cell proliferation of a cell suspension *in vitro* of cells obtained from rat embryos and fetuses, at least up to the 15th day of pregnancy, is accelerated by 2-amino-4-hydroxy-7-methyl pteridine and cancer blood serum and inhibited by xanthopterin and normal human blood serum.

3. The rate of cell proliferation of a cell suspension *in vitro*, of cells obtained from rat young at the time of birth, is accelerated by xanthopterin and normal human blood serum and inhibited by 2-amino-4-hydroxy-7-methyl pteridine and cancer blood serum.

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Spectrophotometric Method for Assay of Serum Antiprotease: Clinical Applications.[*]

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Materials. 1. Trypsin[‖] solution 70 mg% dissolved in veronal buffer, shaken well and filtered, kept cold until used and prepared daily.

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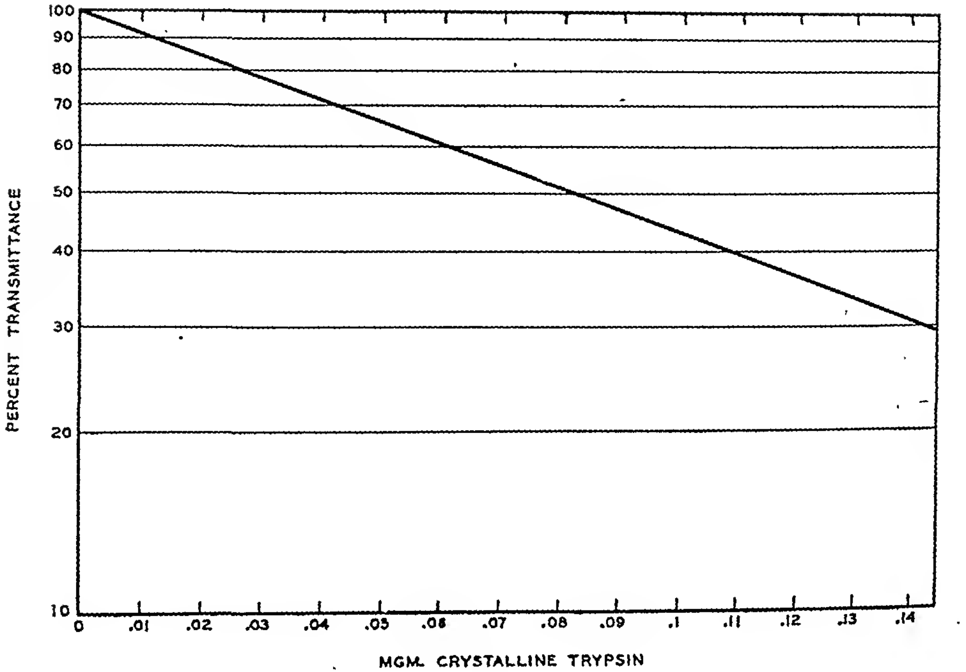


FIG. 1.

acetic acid are added to each tube without shaking and they are left at room temperature for 15 minutes.

The contents of each tube are then filtered through Whatman No. 3 filter paper. When filtration has been completed, a 2.5 ml aliquot of each filtrate is added to 5 ml of NaOH in a 50 ml Erlenmeyer flask. 1.5 ml of diluted phenol reagent is then added at definite time intervals and the contents of the flask are transferred to a standard cuvette. Ten minutes are allowed for color development, when the samples are read at the same time interval against the fibrinogen blank set at 100% transmittance as a reference. A Coleman Spectrophotometer Model 14, set at a wave length of 675 mμ, was employed.

Calculations. The % T (transmittance) readings of uninhibited trypsin and each serum dilution are converted to crystalline trypsin equivalents (CTE) by use of a conversion graph (Fig. 1). The equivalent number of milligrams of crystalline trypsin inhibited by each serum sample is then determined by subtraction of its CTE from the average CTE of the 2 uninhibited trypsins. Tests performed over a period of four weeks have shown that serum of the average patient with no apparent disease inhibits 0.0167 mg of crystalline trypsin. This figure may be used for statistical purposes; however, it is not dependable for individual daily tests because of variations in the reagents, which are prepared daily. Any daily variations are proportional for normal individuals and for those with malignant neoplasia or other diseases. For this reason at least one normal control serum is analyzed with each daily group and the unknowns are reported as % of the normal arbitrarily fixed at 100%.

Results. Tests were performed on 250 samples of blood from patients with various diseases and control patients without evident disease. The results are essentially the same with the two methods (clot formation and spectrophotometric). Although individual cases do not coincide exactly, definite differences occur but rarely. A statistical analysis**

** The statistical analysis was made by Mrs. Sylvia Johnson of the Department of Public Health and the Department of Surgery (Oncology), Yale University School of Medicine, assisted by Mr. David Votaw of the Department of Mathematics, Yale University.

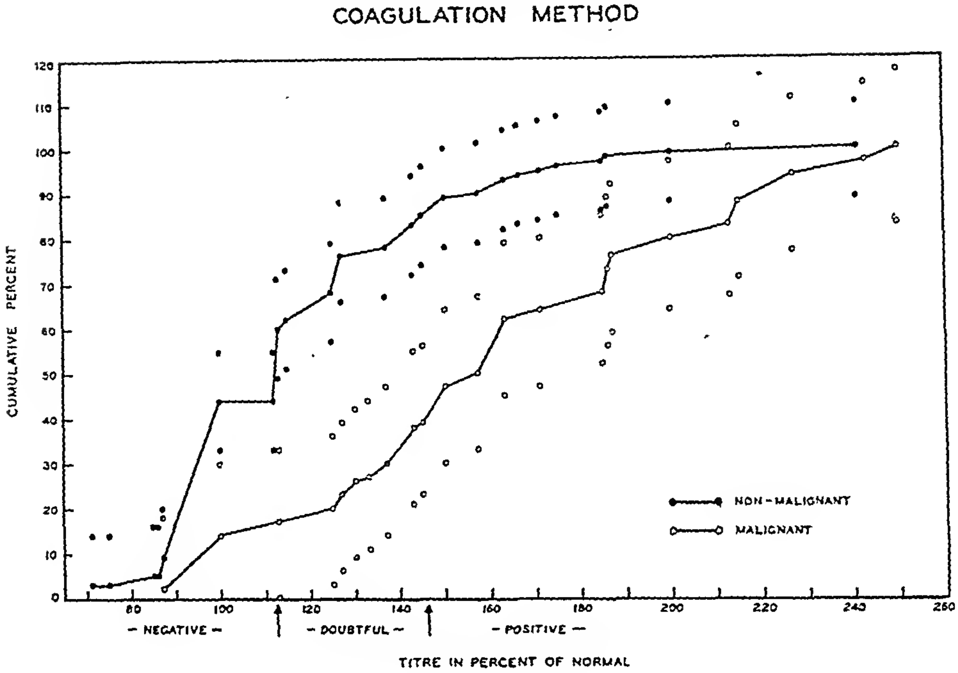


Fig. 2.

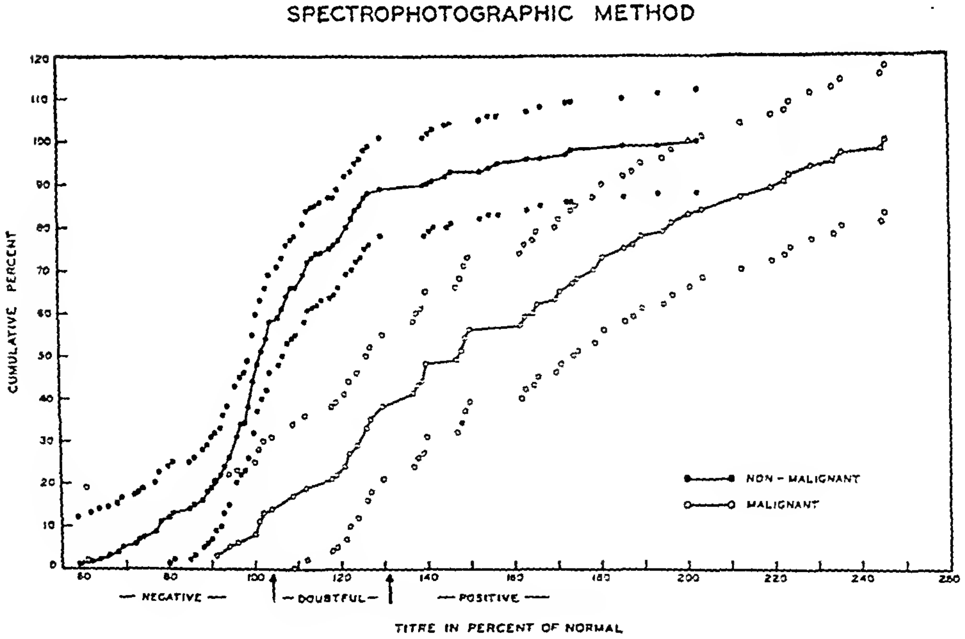
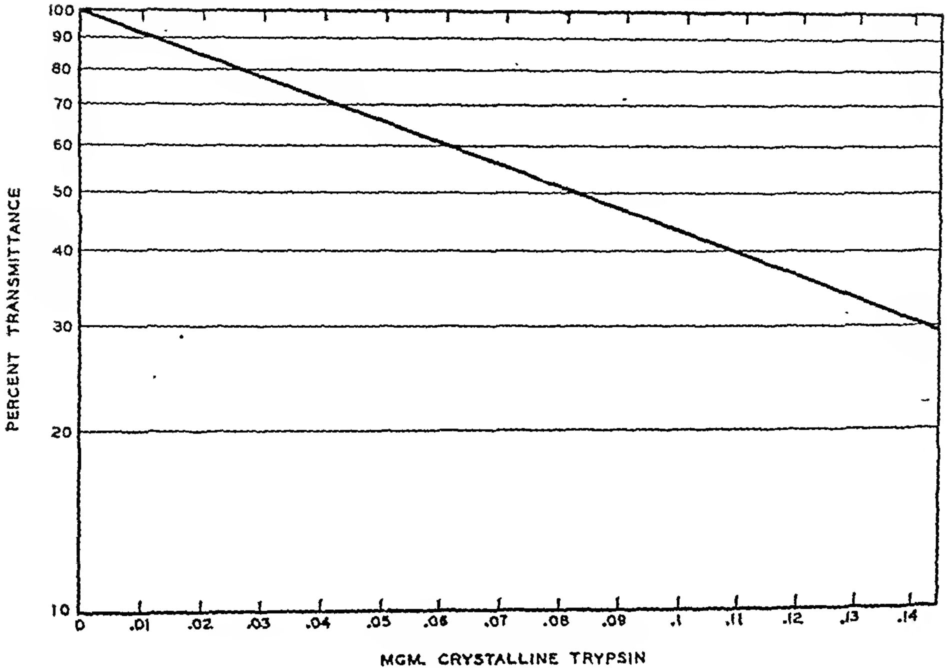


Fig. 3.

of the results in relation to the presence or absence of malignant tumor in the patients studied revealed that the results are comparable (Fig. 2 and 3). This analysis of these cases revealed that using a normal control at 100%, a titre below 112.5% for the clot formation method and below 103% for the spectrophotometric method could be consid-



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Results. Tests were performed on 250 samples of blood from patients with various diseases and control patients without evident disease. The results are essentially the same with the two methods (clot formation and spectrophotometric). Although individual cases do not coincide exactly, definite differences occur but rarely. A statistical analysis**

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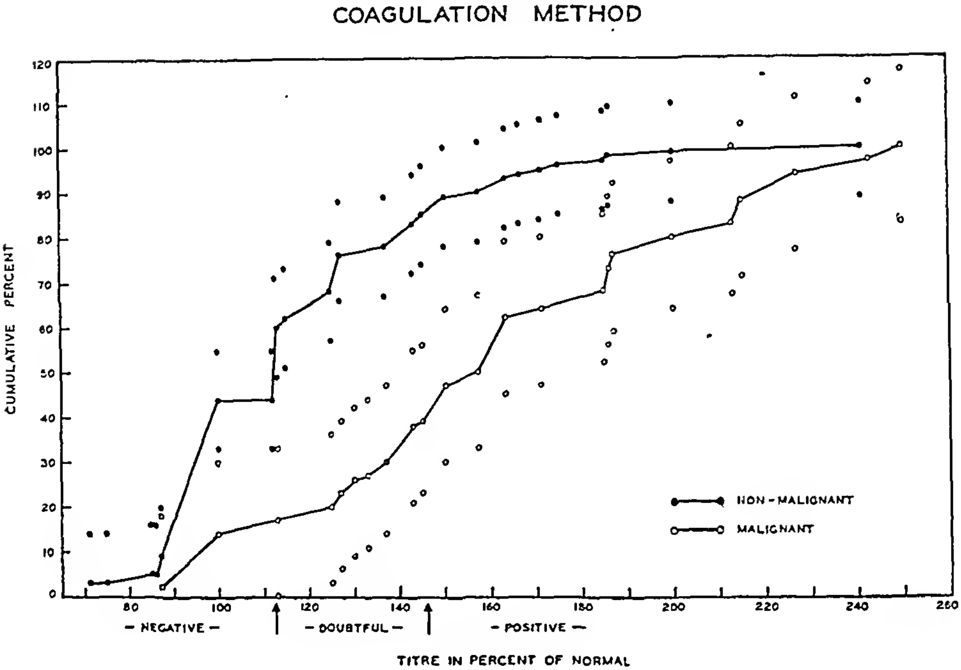
COAGULATION METHOD

FIG. 2.

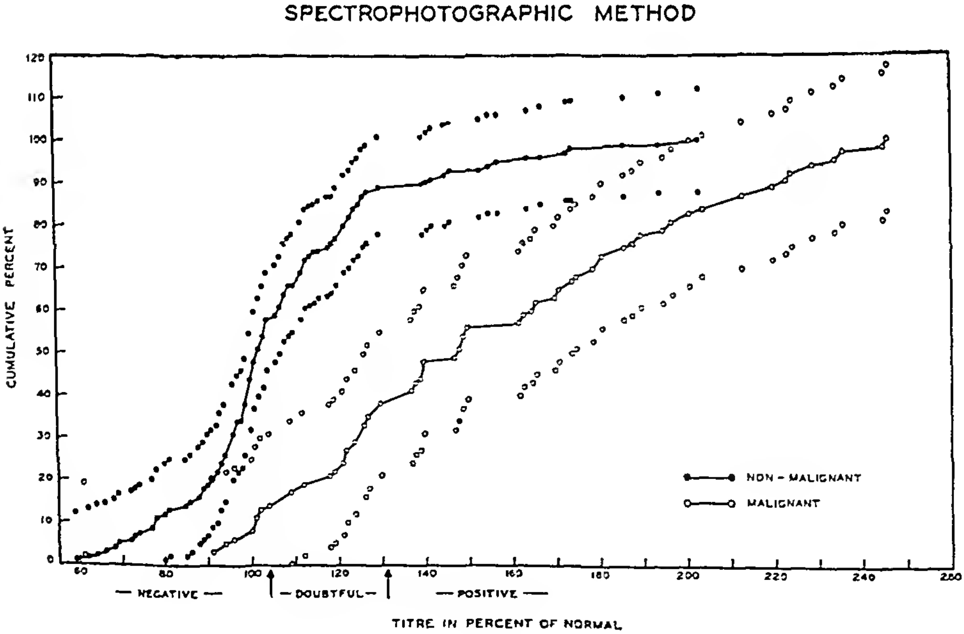
SPECTROPHOTOGRAPHIC METHOD

FIG. 3.

of the results in relation to the presence or absence of malignant tumor in the patients studied revealed that the results are comparable (Fig. 2 and 3). This analysis of these cases revealed that using a normal control at 100%, a titre below 112.5% for the clot formation method and below 103% for the spectrophotometric method could be considered negative with a possible error of 5%, and that a titre above 146% for the clot formation method and above 130% for the spectrophotometric method could be considered as indicative of malignant neoplasia with a possible error of 5%. With the clot formation method, titres between 112.5% and 146% and with the spectrophotometric method titres between 103% and 130% must be considered of doubtful significance.

These results indicate that this spectrophotometric method may be as satisfactory for the detection of patients with malignant neoplasia as the previously reported method.

False positive results with the spectrophotometric method fall in the same groups as with the clot formation method, *i.e.* those patients with acute infectious diseases, especially streptococcal infections, those with advanced tuberculosis of the lungs and those recently subjected to major operations.

Summary. A spectrophotometric method of assay of antiproteolytic material in the serum, using inhibition of trypsin digestion of fibrinogen, has been described. Results obtained in 250 sera by this method have been analyzed statistically and have been found to show a close correlation with the clot formation method.

17028

Microphonic Manometer for Indirect Determination of Systolic Blood Pressure in the Rat.*

MEYER FRIEDMAN AND S. CHARLES FREED.

From Harold Brunn Institute for Cardiovascular Research, Mount Zion Hospital, San Francisco.

Although the plethysmographic method for the indirect measurement of systolic pressure in the rat[1] has been improved by modifications devised later by other workers,[2-5] it has not been adopted universally. This has been due primarily to technical difficulties and errors encountered in any method employing plethysmographic changes for determination of blood pressure.

We have devised a method, however, of obtaining the systolic blood pressure of the rat which is similar in principle to that employed in the indirect measurement of systolic blood pressure in man,—namely, the detection of sound at the exact time that the arterial pressure of the caudal artery exceeds the pressure of the occluding cuff. This method was found to be accurate, simple, and rapid.

The apparatus consists essentially of a carbon type of microphone (3 cm in diameter) to the diaphragm of which is attached a thin copper trough (1.5 cm in length and 0.6 cm in diameter). The microphone is connected to a specially designed, low frequency (100 c.p.s.) sound amplifier† operated by direct current supplied by three dry cells. This amplifier affords a voltage gain of approximately 3000. The energy changes obtained by the amplifier may be detected by ordinary earphones or oscilloscope.

In order to take blood pressure readings, a

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* Aided by a grant from the United States Public Health Service.

[1] Williams, J. R., Jr., Harrison, T. R., and Grollman A., *J. Clin. Invest.*, 1939, **18**, 373.

[2] Kempf, G. F., and Page, I. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1192.

[3] Proskauer, G. G., Neumann, C., and Graef, I., *Am. J. Physiol.*, 1945, **143**, 290.

[4] Sobin, S. S., *Am. J. Physiol.*, 1946, **146**, 179.

[5] Skeggs, L. T., Jr., and Leonards, J. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 294.

† Further details of the construction of this apparatus and the electrical circuit will be furnished by the authors upon request. The microphonic manometer itself may be obtained from Charles Calvert of the Pacific Industrial Electronics Company, San Francisco, Calif.

rat is placed for 10 minutes in a wood box (55 x 25 x 25 cm) having sliding doors at each end and a glass top. The temperature of the box is maintained at approximately 39°C. The tail of the rat is led through a 10 mm pressure cuff and the segment of the tail immediately distal to the cuff is placed in the trough. The pressure of the cuff is raised to a point above the expected blood pressure and slowly released. At the point at which the cuff pressure becomes less than the caudal arterial pressure, one *immediately* hears in the ear phones a rhythmical succession of sounds reflecting at exactly the same rate the transmitted pulsatile variations of the caudal artery. If the amplifier is connected to an oscilloscope, one sees at this same critical point an intense and abrupt change in the contour and frequency of the preceding waves. It should be stressed that the regularity and actual rate of these sounds do not allow their confusion with any possible extraneous movements of either the body or tail of the rat. As the cuff is deflated further, the sounds attain their greatest intensity at the expected diastolic level, although this increase in intensity is too gradual to allow precise determination of the diastolic pressure. The sounds continue even with the cuff completely deflated. No preliminary venous drainage of the tail is necessary as the method does not depend upon gradual increase in tail volume but upon the almost instantaneously transmitted pulsatile variations which occur when the cuff pressure is overcome. Blood pressure determinations can be made at intervals of 15 to 30 seconds.

Results. A. *Blood Pressure of the Normal Rat* (1) *Unanesthetized Rats.* Twelve albino rats weighing between 150-200 g were given 0.02 mg of Intracostrin (Squibb) per 100 g of body weight. This amount of curare, although insufficient to produce paralysis, did subdue the rats sufficiently that they remained relatively quiet when wrapped loosely in a cloth binder. The average systolic blood pressure of these rats as determined by the microphonic manometer was 98 mm of Hg. (Range: 82 to 120 mm Hg). Repeated daily determinations of the same animal rarely deviated more than 4-10 mm of Hg from the initial reading. These determinations are comparable to those obtained by previous investigators.[1,2]

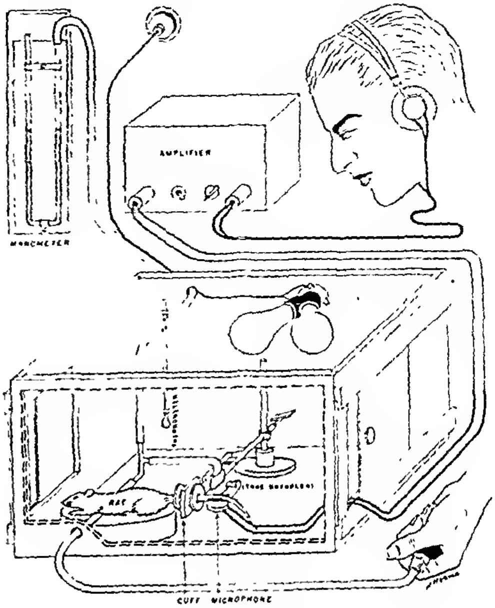

FIG. 1.

Drawing depicts entire apparatus. The rat is placed on a pad of sponge rubber. The cuff is inflated by hand bulb and air pressure is measured by manometer. As the air is released, the cuff pressure falls below that in tail artery and the pulsatile vibration instantly detected by the microphone is amplified into rhythmic audible signals heard by the observer.

(2) *Anesthetized Rats.* Twenty rats similar to those above were anesthetized by intraperitoneal injection of pentobarbital sodium. The average systolic blood pressure of these rats was 90 mm of Hg (Range: 70 to 104 mm of Hg). Later subsequent determinations of the same animals did not deviate more than 4-8 mm of Hg from the initial value.

B. *Blood Pressure of Rat after Injection of Epinephrine and Renin.* Five anesthetized rats whose average initial pressure was 91 mm of Hg (Range: 82 to 102 mm of Hg) were given 0.2 mg of epinephrine in oil by subcutaneous injection. Thirty minutes later, the average pressure was 180 mm of Hg (Range: 144 to 208 mm of Hg).

Twelve anesthetized rats were given one mg of purified hog renin by intravenous injection. The pressure of each rat was determined immediately and continuously after the injection. It was observed that the average pressure of 88 mm of Hg before injection increased to a maximal average of 170 mm of Hg approximately two minutes after injection. Similar to the pressor response of the dog to renin, the pressures of the injected rats gradually fell after maintaining a plateau for approximately five minutes after injection. The pressures usually reached the control levels about 20 minutes after injection. Again similar to the dog, the rat was observed to develop tachyphylaxis rather quickly to injections of renin. Thus successive injections of the same amount of renin effected progressively smaller and more evanescent pressor effects and the third or fourth successive injection usually caused little or no rise in pressure.

Summary. The ability of the microphonic manometer to measure rapidly and simply not only the pressure of the intact rat but also of the rat made hypertensive by the injection of the vasoconstrictor substances, epinephrine and renin, indicates that it might be of considerable usefulness in studies necessitating frequent or successive blood pressure determinations in the rat.

The authors would like to express their appreciation for technical assistance rendered by Vivian Seay.

17029

Kinetics of Distribution of Inulin Between Two Body Water Compartments.

MARIO GAUDINO.* (Introduced by Homer W. Smith.)
From the Department of Physiology, New York University College of Medicine, New York City.

The kinetics of distribution of inulin between the intravascular and the interstitial compartments after its introduction into the circulatory system is of interest in relation to the use of this substance as a measure of the extracellular volume.[1,2]

The present paper concerns the distribution of any substance with the properties of inulin between two fluid systems representing schematically the intravascular and interstitial compartments of the body.

Let V_1 be the volume[†] of the intravascular compartment (I) and V_2 the volume of the interstitial compartment (E), both of which will be assumed to remain constant during the experimental procedure. It is further supposed that the rate of mixing of the substance in each of these compartments is rapid compared to simple diffusion. If the concentration of the substance in I is represented by $c_1(t)$, the concentration in E by $c_2(t)$, the concentration in the urine (assumed to be the only route of elimination) by $c_3(t)$, and if the flow (q_1) of the liquid that is being interchanged between the two compartments is assumed to be constant, q_2 being the urine flow, then, $c_1(t)V_1$ is the amount of substance accumulated in I at any instant, $c_2(t)V_2$ the amount accumulated at any instant in E, $[c_1(t)—c_2(t)]\ q_1$ the rate of interchange of the substance between I and E, and finally, $c_3(t)\ q_2$ is the rate of elimination. In the case under consideration, the substance (inulin) is eliminated only by glomerular filtration, and if the renal clearance at any instant is q_3, then $c_3(t)\ q_2 = c_1(t)\ q_3$. The substance will be introduced into the system in the form of a chemical solution with a rate CQ, where C is its concentration and Q is the rate of infusion, both C and Q being kept constant experimentally.

Under these conditions, the rate of accumulation of the substance in the vascular compartment will be:

$$V_1 \frac{dc_1}{dt} = QC - q_1(c_1 - c_2) - q_3c_1 \qquad (1)$$

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* Dazian Foundation Fellow.

[1] Gaudino, M., Schwartz, I. L., Levitt, M. F., PROC. SOC. EXP. BIOL. AND MED., 1948, 68, 507.

[2] Gaudino, M., Levitt, M. F., *Am. J. Physiol.*, 1949, in press.

[†] Volumes are expressed in cc of fluid, independently of any volume effects of protein, flows in cc per minute, concentrations in mg per cc.

and the rate of accumulation in the interstitial compartment:

$$V_2 \frac{dc_2}{dt} = q_1(c_1 - c_2) \qquad (2)$$

Equation (1) and (2) form a system of linear differential equations the solutions of which are:

$$c_1(t) = A\,e^{\lambda t} \qquad (3)$$

$$c_2(t) = B\,e^{\lambda t} \qquad (4)$$

Substituting (3) and (4) into (1) and (2), after appropriate differentiation:

$$V_1V_2\lambda^2 + (V_1q_1 + V_2q_1 + V_2Q_3)\lambda + q_1q_3 = 0 \qquad (5)$$

(5) being the characteristic equation corresponding to the system (1) (2). The roots λ_1 and λ_2 of (5) will be:

$$-(\lambda_1,\lambda_2) = (\beta,\alpha) \qquad (6)$$

and

$$(\beta,\alpha) = \frac{1}{2V_1V_2}\Big[\,(V_1q_1 + V_2q_1 + V_2q_3) \pm \sqrt{(V_1q_1 + V_2q_1 + V_2q_3)^2 - 4q_1q_3V_1V_2}\,\Big] \qquad (7)$$

Depending on the manner in which the substance is introduced in the organism, different solutions of this system of differential equations will be obtained. Three cases will be considered: (a) the substance will be introduced in the form of a rapid and single injection; (b) it will be introduced at a constant rate until uniform distribution between the intravascular and interstitial compartments has been reached; (c) the behavior of the substance will be studied after the cessation of the infusion required to maintain the steady state represented by (b).

(a) *Single injection.* In the case in which a single injection is given (50 cc in about 1 minute) the substance can be assumed to be introduced instantaneously into the system. Then, the following solution holds:

$$c_1(t) = A\,e^{-\alpha t} + B\,e^{-\beta t} \qquad (8)$$

where A and B are integration constants which are calculated from (1) and (8). Their values are:

$$A = \frac{\beta c_1(o)}{\beta - \alpha} \qquad (9)$$

$$B = -\left(\frac{\alpha c_1(o)}{\beta - \alpha}\right) \qquad (10)$$

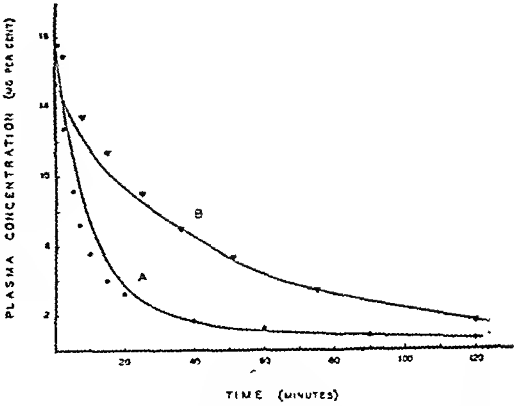

FIG. 1.

Rate of disappearance of inulin from the plasma after a single injection (A) and after a constant infusion (B). The curves are calculated from equations (8) and (17) respectively, the values $\alpha = 0.01$, $\beta = 0.10$, $A = 2.0$, $B = 15.2$, $P = 12.4$, $R = 3.0$, being obtained by trial and error. The two curves were obtained in the same dog.

$c_1(o)$ representing the concentration of the substance in I at $t = 0$.

Equation (8), with the appropriate substitutions, gives the solution of this case and permits the calculation of the rate of disappearance of the substance from the intravascular compartment.

To test this equation, single injections of inulin were given to 3 normal dogs and the plasma concentration was determined at frequent intervals and related to time. The results were identical in all of the experiments, the observed curve corresponding closely with the theoretical curve calculated by giving values to the constants in (8), (Fig. 1A).

(b) *Constant infusion.* If the rate of introduction of the substance is the same as the rate of elimination (a condition attained by constant intravenous infusion), then,

$$q_3c_1 = QC \text{ or } c_1 - \frac{QC}{q_3} = 0 \qquad (11)$$

Letting

$$c_1 - \frac{QC}{q_3} = y, \qquad (12)$$

the general solution of (5) is

$$y = K\,e^{-\alpha t} + D\,e^{-\beta t} \qquad (13)$$

and hence

$$c_1(t) = \frac{QC}{q_3} + K\,e^{-\alpha t} + D\,e^{-\beta t} \qquad (14)$$

termined immediately and continuously after the injection. It was observed that the average pressure of 88 mm of Hg before injection increased to a maximal average of 170 mm of Hg approximately two minutes after injection. Similar to the pressor response of the dog to renin, the pressures of the injected rats gradually fell after maintaining a plateau for approximately five minutes after injection. The pressures usually reached the control levels about 20 minutes after injection. Again similar to the dog, the rat was observed to develop tachyphylaxis rather quickly to injections of renin. Thus successive injections of the same amount of renin effected progressively smaller and more evanescent pressor effects and the third or fourth successive injection usually caused little or no rise in pressure.

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17029

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Under these conditions, the rate of accumulation of the substance in the vascular compartment will be:

$$V_1 \frac{dc_1}{dt} = QC - q_1(c_1 - c_2) - q_3c_1 \qquad (1)$$

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[*] Dazian Foundation Fellow.

[1] Gaudino, M., Schwartz, I. L., Levitt, M. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 507.

[2] Gaudino, M., Levitt, M. F., *Am. J. Physiol.*, 1949, in press.

[†] Volumes are expressed in cc of fluid, independently of any volume effects of protein, flows in cc per minute, concentrations in mg per cc.

17030

Biological Oxidation of Phospholipids by Rat Liver Homogenates.[*]

Paul W. O'Connell and Elmer Stotz. (Introduced by A. L. Dounce.)

From the Department of Biochemistry, School of Medicine and Dentistry, The University of Rochester, Rochester, N. Y.

The normal saturated C_4 to C_{18} fatty acids have been shown to be oxidized by homogenized rat liver preparations in the presence of adenosine triphosphate (ATP) and cytochrome c,[1] with acetoacetate as a product of the reaction.[2] Presumably this reaction represents fatty acid oxidation *in vivo*, but the concentration of free fatty acids in animal tissues is actually very low.[3] Hence other lipids may also serve as the source of fatty acid for oxidation, and among these the phospholipids have been strongly implicated as intermediates in fat metabolism.[4] Consequently, in the work reported here, measurements were made of the oxygen consumption and acetoacetate production from phospholipids, utilizing the type of enzyme preparation which oxidizes free fatty acids.

Experimental. Substrates. The petroleum ether-soluble, acetone-insoluble fraction of beef brain lipid was prepared and found to contain 3.3% phosphorus and to have an iodine number of 85.5. It was stored in petroleum ether, under nitrogen, at 5°. The beef lung hydrolecithin was generously supplied by Dr. S. J. Thannhauser. Emulsions of these compounds were prepared by suspending 80 mg of the phospholipid in 10 ml of 0.1 M phosphate buffer, pH 8.0, and homogenizing the mixture in a Potter-Elvehjem glass homogenizer. A 0.01 M sodium octanoate solution, used to test the activity of the enzyme preparations for fatty acid oxidation, was prepared by neutralizing redistilled *n*-octanoic acid with the required amount of NaOH and adjusting the pH to 7.0-7.5.

Reagents. Cytochrome c was prepared from beef heart according to the method of Keilin and Hartree.[5] The stock solutions used varied from 1.3 to 2.0 × 10^{-4} M. Adenosine triphosphate was prepared from rabbit muscle according to the method of Dounce *et al.*[6]

Analytical methods. For the measurement of oxygen consumption, Warburg flasks of about 20 ml total volume were used. The flasks were constructed with two side arms. All experiments were run at 30° and in all cases the gas phase was air. The center well of each flask was equipped with a filter paper roll and 0.2 ml of 20 per cent KOH. Acetoacetic acid was determined by distilling a metaphosphoric acid filtrate of the incubated samples and determining the acetone by the vanillin method of Alyea and Backström.[7] The detailed procedure has been described by Witter and Stotz.[8]

Liver Homogenate. White rats, which had been starved overnight, were decapitated and bled. The liver was removed and homogenized in the manner described by Lehninger,[1] using an all-glass homogenizer which has been described by Dounce.[9]

The Oxidation of Beef Brain Phospholipid.

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[*] This research was supported by grants from the Nutrition Foundation, Inc., The Sugar Foundation, Inc., and the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

[1] Lehninger, A. L., *J. Biol. Chem.*, 1945, **157**, 363.

[2] Lehninger. A. L., *J. Biol. Chem.*, 1945, **161**, 413.

[3] Fairbairn, D., *J. Biol. Chem.*, 1945, **157**, 645.

[4] Sinclair, R. G., *Physiol. Rev.*, 1934, **14**, 351.

[5] Keilin, D., and Hartree, E. F., *Proc. Roy. Soc., London. B*, 1937, **122**, 298.

[6] Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R., and Freer, R., *J. Biol. Chem.*, 1948, **174**, 361.

[7] Alyea, H. N., and Backström, H. J., *J. Am. Chem. Soc.*, 1929. **51**, 90.

[8] Witter, R. F., and Stotz, E., *J. Biol. Chem.*, 1948, **176**, 501.

[9] Dounce, A. L., and Beyer, G. T., *J. Biol. Chem.*, 1948, **174**, 859.

in which α and β are defined by (7) and K and D are constants of integration with the following values:

$$K = \frac{QC - V_1\beta\left(\frac{QC}{q_3}\right)}{V_1\,(\beta - \alpha)} \tag{15}$$

$$D = -\left[\frac{QC - V_1\alpha\left(\frac{QC}{q_3}\right)}{V_1\,(\beta - \alpha)}\right] \tag{16}$$

that can be replaced in (14).

According to equation (14) when t becomes infinite the concentration of the substance in the blood will depend only on the rate of infusion and on the rate of glomerular filtration. As both are supposed to be constant, the plasma concentration also becomes constant. Further, since it was assumed that the rate of mixing of the substance in both compartments is rapid, the concentration in the interstitial space can be considered the same as the concentration in the plasma water.

Experimentally, this equilibrium has been attained in 2 hours in the dog.[2] In both cases the blood level follows the general pattern described by (17).

(c) *After cessation of constant infusion.* The general solution of (5) after a constant infusion has been discontinued is:

$$c_1(t) = P\,e^{-\alpha t} + R\,e^{-\beta t} \tag{17}$$

where P and R are integration constants with the values:

$$P = \frac{\beta V_1 c_1(n) - q_3 c_1(n)}{V_1\,(\beta - \alpha)} \tag{18}$$

$$R = -\left(\frac{\alpha V_1 c_1(n) - q_3 c_1(n)}{V_1\,(\beta - \alpha)}\right) \tag{19}$$

Here $c_1(n)$ is the concentration in the plasma water at the moment of interrupting the infusion when $t = n = 0$. It will be observed that the only difference between (8) and (17) lies in the values of the integration constants.

The decrement in plasma concentration in relation to time was studied in 3 normal dogs after the interruption of a constant infusion of 2 hours duration. The results obtained were similar in the three cases and again show good correspondence with the theoretical curve obtained by giving values to P and R in (17) (Fig. 1B).

The difference between the curves obtained (a) after a single injection and (b) after constant infusion to equilibrium shows that uniform distribution is never reached after a single injection.

Further, as during constant infusion, the condition stated in (11), namely that $CQ = q_3c_1$, is fulfilled at the moment of interruption the amount of substance (Z) contained in both I and E will disappear from the body at a rate expressed only by q_3c_1. The total amount of inulin excretion will then be given by:

$$Z = q_3\int_0^\infty c_1(t)\,dt \tag{20}$$

Introducing (17) with the appropriate substitution in (20), integrating within the limits indicated, and substituting the values of α and β from (7), the result is:

$$(V_1 + V_2) = \frac{Z}{c_1(n)} \tag{21}$$

where $V_1 + V_2$ is the total volume of the extracellular space. This volume can then be calculated by dividing the total amount (Z) of inulin excreted since the interruption of the infusion, by the concentration $c_1(n)$ of this substance in the blood at the moment of interruption, as previously reported.[1,2] The volume of distribution of inulin thus measured corresponds to 19 per cent of the body weight in the dog[2] and to 16 per cent in man.[1]

Summary. Comparison of the rate of disappearance of inulin from the plasma after a single injection and after prolonged constant infusion yields data which, on mathematical analysis, conform with the assumption that in the latter circumstance, inulin is uniformly distributed throughout some fixed volume of body fluid, presumably the extracellular fluid.

The author is deeply indebted to Dr. Domingo M. Gomez for his valuable advice in the consideration of this problem.

of the phospholipid molecule, hydrolecithin should not be susceptible to auto-oxidation.

The hydrolecithin was used as a substrate with the enzyme preparation previously described. The results of typical experiments are recorded in Table II.

Discussion. Since acetoacetic acid is not metabolized by the homogenized rat liver preparation, and there is no inhibition of acetoacetic acid formation when octanoate and phospholipid are added to the same preparation, the failure of the oxidizing phospholipid to produce acetoacetic acid indicates that free fatty acids are not liberated from the phospholipid in the preparation employed.

It was found that the rat liver homogenate was able to dehydrogenate phospholipid in the presence of ATP, as measured by the Thunberg technic. The aerobic reaction, however, did not result in an increased phospholipid iodine number, and it was found impossible to determine what changes might have occurred in the fatty acids of the phospholipid as a result of the oxidation. Nevertheless, the fact that both free fatty acids and phospholipid are oxidized by the same enzyme preparation prompts the suggestion that this new oxidative reaction of phospholipid is somehow related to intermediary fatty acid metabolism.

Summary. Beef brain phospholipid and beef lung hydrolecithin are oxidized by rat liver homogenates in the presence of adenosine triphosphate. Although this preparation oxidizes free fatty acids with the production of acetoacetic acid, the phospholipid oxidation does not result in such a product.

17031

Effects of Glucose Fermentation Products on Determination of Mannitol by Periodate-Titrametric Method.

A. B. KENDRICK, W. P. SWISHER, AND R. A. FORREST. (Introduced by R. W. Keeton.)
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When mannitol clearance and maximal glucose reabsorptive capacity are measured simultaneously in studies of renal function,[1,2] it becomes necessary to determine mannitol in the presence of high concentrations of glucose in both plasma and urine. Satisfactory recoveries of mannitol under these conditions have been reported by others,[2] but certain discrepancies have been noted by us. This report deals with a) the extent of interference of glucose fermentation products with the determination of mannitol, b) a correction factor, and c) the nature of the interfering substance.

Glucose was added to water in varying concentrations from 0 to 1250 mg %. The increases were made in steps of 50 mg %. The solutions were then analyzed for mannitol by the method of Smith, Finkelstein, and Smith[3] except that $CdSO_4$-NaOH[4] was used instead of $ZnSO_4$-NaOH as the precipitating reagent, and the time of fermentation was extended to 2 hours. The method entails fermentation of glucose from the samples by yeast, removal of yeast by centrifugation, precipitation of the proteins, oxidation of the mannitol in the filtrate with periodic acid, and the determination of the excess periodic acid, together with the iodic acid formed, by titration with sodium thiosulfate. All of the filtrates used in our determinations were analyzed quantitatively for glucose[5] to make cer-

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[1] Smith, H. W., *J. Mt. Sinai Hospital*, 1943-44, **10**, 59.

[2] Klop, C., Young, N. F., Taylor, H. C., Jr., *J. Clin. Invest.*, 1945, **24**, 117.

[3] Smith, W. W., Finkelstein, N., Smith, H. W., *J. Biol. Chem.*, 1940, **135**, 231.

[4] Fugita, A., Iwatake, D., *Biochem.*, 1931, **242**, 43.

TABLE I.
Oxidation of Beef Brain Phospholipid.

Substrate	ATP*	Oxygen consumption, cmm	Acetoacetic acid, μM
Endogenous†	+	24	1.95
Octanoate	+	85	4.50
Phospholipid	+	75	1.80
		104	1.80
Phospholipid	—	0	1.00
Endogenous	+	13.6	1.50
Octanoate	+	110	3.60
Phospholipid	+	109	1.55
″	—	3.2	0.75
Endogenous	+	8	1.40
Octanoate	+	51	2.25
Phospholipid	+	73.1	1.45
		103	1.50
″	—	13.5	1.05

* When absent, replaced by equal volume of water in side arm.
† Substrate replaced by equal volume of water.

TABLE II.
Oxidation of Beef Lung Hydrolecithin.

Substrate	ATP	Oxygen consumption, cmm	Acetoacetic acid, μM
Endogenous	+	114	—*
Hydrolecithin	+	182	—
″	—	27.0	—
Endogenous	+	41.0	1.35
Octanoate	+	165	6.70
Hydrolecithin	+	167	1.40

* Not determined.

The main compartment of each Warburg flask contained 0.4 ml of 0.1 M phosphate buffer, pH 7.7, 0.2 ml of cytochrome c, and 0.1 ml of 0.1 M magnesium chloride. One side arm of each flask contained 0.4 ml of 0.01 M ATP solution, adjusted to pH 7.5. The other side arm contained 0.4 ml of substrate solution. Just before equilibration, 0.5 ml of liver homogenate were added to the main compartment of each flask. The equilibration period was 5 minutes, at which time the taps were closed and the contents of the side arms tipped. The oxygen uptake was nearly linear through a 30 minute period of measurement and the results reported are expressed in terms of a total 30 minute oxygen consumption. After the measurements of the oxygen consumption, the contents of the flasks were analyzed for acetoacetic acid. The results of typical experiments are recorded in Table I.

The homogenized rat liver preparation oxidized both free fatty acids and phospholipid in the presence of ATP. Only the oxidation of the free acid resulted in the production of acetoacetic acid in amounts significantly greater than was produced by preparations to which no substrate was added. The necessity of ATP for phospholipid oxidation and the rate of the oxygen consumption suggested that the oxidation was of a true biological nature, rather than an auto-oxidation.

The Oxidation of Beef Lung Hydrolecithin. The above conclusion was supported by results obtained through the use of beef lung hydrolecithin as a phospholipid substrate. Both the fatty acids of the molecule are palmitic acid.[10] Since auto-oxidation is believed to involve the reaction of oxygen with the unsaturated acids

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[10] Thannhauser, S. J., Benotti, J., and Boncoddo, N., *J. Biol. Chem.*, 1946, **166**, 669.

17032 P

Serum Level of Protein Bound Radioactive Iodine (I^{131}) in the Diagnosis of Hyperthyroidism.*

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With the technical assistance of Barbara Seamon.
(Introduced by Herrman L. Blumgart.)

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It is generally agreed that the blood level of protein bound ("hormonal") iodine is a measure of thyroid activity; in most patients with thyrotoxicosis the plasma protein bound iodine levels are above 8-10 γ %.[1-4] The chemical determination of plasma protein bound iodine is, however, difficult and laborious and despite its importance is not widely used. Recently, Taurog, Chaikoff, and Entenman[5] have used radioactive iodine (I^{131}) to measure the turnover of plasma protein bound iodine in dogs. It seemed possible that thyroid function might be estimated in man by measuring the concentration of serum protein bound radioactive iodine following an oral dose of I^{131}.

Methods. One hundred and fifty microcuries of I^{131}, carrier free, were administered orally to 20 subjects, aged 24 to 65 years; 16 were female. Ten subjects were thyrotoxic; the other 10 were euthyroid. The thyrotoxic subjects had the characteristic symptoms and signs. The basal metabolic rates ranged from +15 to +60, and averaged +34%. The circulation time, blood cholesterol, the body retention of I^{131} as measured by urinary excretion,[6] and the I^{131} uptake in the thyroid measured by external counts[7] were consistent in each instance with the clinical diagnosis of thyrotoxicosis. The other 10 subjects were euthyroid by these criteria. The basal metabolic rate ranged from —10 to +15 and averaged +2%.

The I^{131} was given 3 hours after a light breakfast. Twenty-four hours later, 10 cc of venous blood was obtained from each subject and the serum separated.

Procedure for determination of protein bound radioactive iodine. A. 1.0 cc serum was pipetted into a previously weighed glass boat measuring 25 mm in diameter and 7 mm deep.

B. The protein bound I^{131} was separated by the method of Chaikoff *et al.*[8] modified as follows:

(1) 1.0 cc serum was pipetted into a small centrifuge tube and 1.0 cc 10% trichloracetic acid (cold) added.

(2) After centrifugation (2500 RPM) for 30 minutes, the supernatant was removed and saved.

(3) The precipitate was washed twice with 2-5 cc cold 5% trichloracetic acid. After each addition of trichloracetic acid and centrifugation, the supernatant fluid was removed. The washings were pooled.

(4) The precipitate was dissolved in 1.0 cc 2 N NaOH and transferred to a weighed glass boat.

C. 1.0 cc of the collected supernatant (inorganic fraction) was adjusted to pH 7.5 and

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[*] This work was carried out under a contract of the Office of Naval Research Atomic Energy Commission and the President and Fellows of Harvard College.

[1] Bassett, A. M., Coons, A. H., and Salter, W. T., *Am. J. M. Sc.*, 1941, **202**, 516.

[2] Salter, W. T., Bassett, A. M., and Sappington, T. S., *Am. J. M. Sc.*, 1941, **202**, 527.

[3] Man, E. B., Smirnow, A. E., Gildea, E. F., and Peters, J. P., *J. Clin. Invest.*, 1942, **21**, 773.

[4] Riggs, D. S., Trans. Am. Assn. Study of Goiter, 1947, pp. 137-144.

[5] Taurog, A., Chaikoff, I. L., and Entenman, C., *Endocrinology*, 1947, **40**, 86.

[6] Freedberg, A. S., Buka, R., and McManus, M. J., *J. Clin. Endoc.*, in press.

[7] Freedberg, A. S., Ureles, A., and Van Dilla, M., *Fed. Proc.*, 1949, **8**, 50.

[8] Chaikoff, I. L., Taurog, A., and Reinhardt, W. O., *Endocrinology*, 1947, **40**, 47.

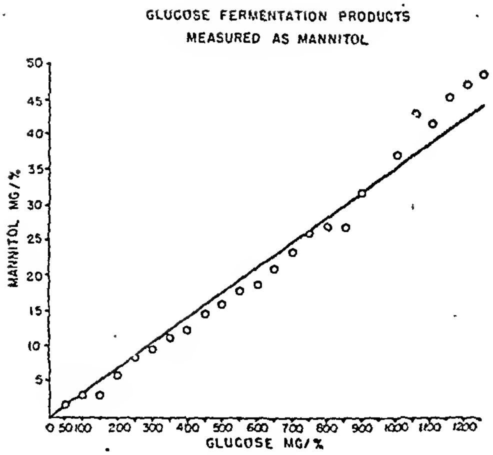

FIG. 1.

tain that the fermentation was complete. The titrations of the filtrates for mannitol were found to increase almost in direct proportion to the quantity of glucose removed. (Fig. 1). An analysis of the results revealed that 3.51% of the glucose fermented was measured as mannitol.

Attempts were made to control the formation of measurable glucose fermentation products by using a constant temperature incubator and increasing the temperature to 37°C. Recovery of apparent mannitol was thus increased to 4.9% of the glucose fermented, indicating that an increased and more variable production of the interfering substance had occurred.

Under optimum conditions for fermentation of glucose by yeast at 25 to 30°C and at a pH 5.6 to 5.8,[6] the glycerol formed is equivalent to 3 to 4% of the glucose destroyed.[7] Periodic acid, which is used for the oxidation of mannitol, will also oxidize the glycerol,[8] each cc of 0.005 N sodium thiosulfate being equivalent to 0.091 mg of mannitol or to 0.093 mg of glycerol. Para-aminohippuric acid is also oxidized by periodic acid, and hence interferes with the determination of mannitol. The method of Barker and Clark[9] prevents this interference, but not the reaction of periodic acid with the fermentation products of glucose.

When the effect of the glucose fermentation products are ignored, the mannitol clearance values determined simultaneously with the maximal glucose reabsorptive capacity are lowered, the degree depending upon the relative amounts of glucose and mannitol in plasma and urine. The amounts vary with the ratio of the mannitol clearance to the maximal tubular glucose reabsorptive capacity, the mannitol clearance value, and the plasma levels of mannitol and glucose. The details of the factors which affect the mannitol clearance are shown in the following equation:

$$\frac{U_M V + ((C_M P_G - T_m G) \times \textit{Glucose Correction})}{P_M + (P_G \times \text{Glucose Correction})} = C_M \text{ Uncorrected Values.}$$

C_M = mannitol clearance, cc/min.
U_M = urine mannitol, mg/cc.
V = urine vol., cc/min.
P_M = plasma mannitol, mg/cc.
P_G = plasma glucose, mg/cc.
$T_m G$ = max. glucose reabsorptive capacity, mg/min.

The mannitol clearance values are always lowered as the concentration of glucose in the urine is less than that of plasma due to the tubular reabsorption of glucose.

Summary. 1. Glucose fermentation products interfere with the periodate-titrimetric method of mannitol determination. 2. Failure to correct for this interference lowers the mannitol clearance values.

The advice of Dr. Robert W. Keeton, the technical assistance of Mr. Edward Eckert, and the generous donation of mannitol by Dr. J. Wm. Crosson of Sharp and Dohme, Inc., are gratefully acknowledged.

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[5] Nelson, Norton, *J. Biol. Chem.*, 1944, **153**, 375.

[6] Harden, Arthur, Alcoholic Fermentation, Longman, Green and Co., 1923, third edition.

[7] Anderson, C. G., An Introduction to Bacteriological Chemistry, Williams and Wilkins Co., 1946, pp. 310, 311.

[8] Bradford, P., Pohle, W. D., Gunther, J. K., Mehlendbacher, V. C., *Oil and Soap*, 1942, **19**, 189.

[9] Barker, Harold G., and Clark, John K., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 120.

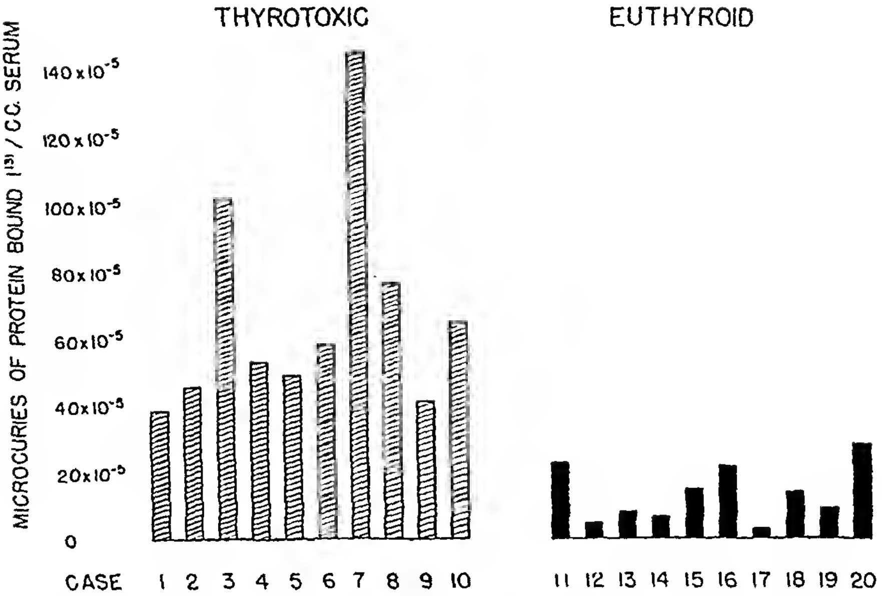

FIG. 1.
Serum Level Protein Bound I^{131}, 24 hours after standard oral dose of 150 μc I^{131}, carrier free, in 10 thyrotoxic and 10 euthyroid subjects.

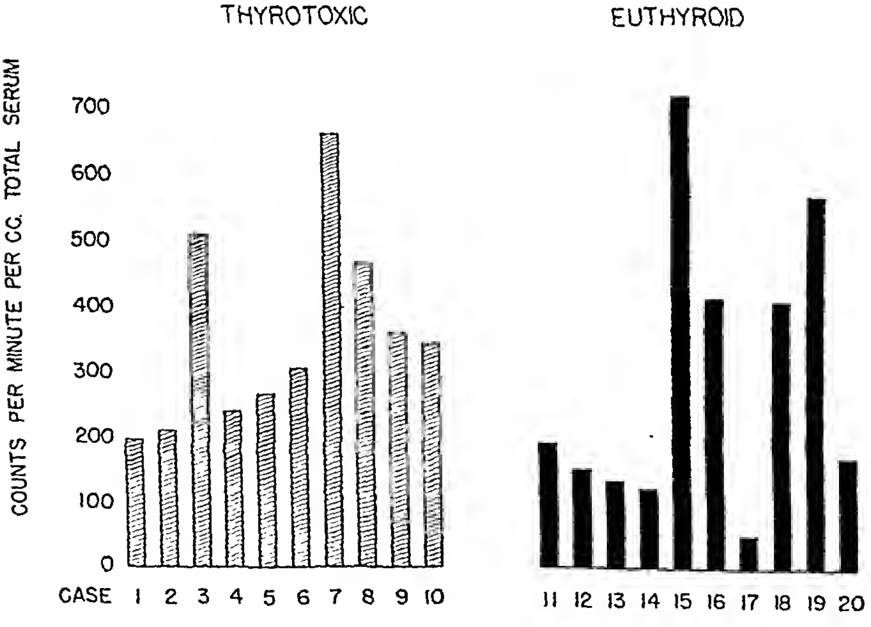

FIG. 2.
Counts/min./cc serum 24 hours after standard oral dose 150 μc I^{131}, carrier free. in 10 thyrotoxic and 10 euthyroid subjects.

after therapeutic doses (4-6 millicuries) showed that the protein bound I^{131} level at 24 hours was 50% higher than at 6 hours. The protein bound I^{131} serum levels at 48 and 72 hours, however, were increased only irregularly over the 24-hour concentration.

TABLE I.

M.S., Age 62; Duodenal Ulcer; Euthyroid. Oct. 13, 1948; 10 a.m., 150 μc. I^{131}, carrier free by mouth. Oct. 14, 1948, 10 a.m., 10 cc venous blood drawn.

	cc	Dry wt, mg	Net counts/min., corrected for mass and dilution	Correction for decay, %	Net counts /min./cc
A. Total serum	1.0	110	242	77	315
B. Precipitate	1.0	89	50	77	65
C. Filtrate	1.0	54	185	77	239

Background 15 counts/min.
From nomogram 0.001 microcurie = 450 cts./min.
B. Precipitate (Protein Bound I^{131}) 65 cts./min. = .00015 μc = 15×10^{-5} μc.

transferred to a previously weighed glass boat.

One drop 10% gelatin (2-3 mg) was added to each cup and the samples evaporated slowly (37 to 40°C) to dryness. The cups were reweighed and the radiation determined with an end window Geiger-Mueller tube (3.2 mg/cm^2). Corrections for mass absorption, dilution, and decay were made. All determinations were extrapolated to the time the sample was obtained.

Calculations. The total serum activity per cc (Table I) should equal the precipitate (protein bound I^{131}) activity, plus the filtrate (inorganic I^{131}) activity. The conversion to microcuries of net counts per minute per cubic centimeter was made by reference to a nomogram. Under these geometric and physical conditions, .001 microcurie I^{131} gave 450 net counts per minute. The reference standard for I^{131} radiation was Bi 210 (half life 22 years).

The following protocol (Table I) is illustrative.

Results. The results are shown in Fig. 1 and 2. In the hyperthyroid patients the serum protein bound I^{131} ranged from 38 to 146 $\times 10^{-5}$ μc/cc (Fig. 1), averaging 68 $\times 10^{-5}$ μc. In the euthyroid patients the serum protein bound I^{131} ranged from 3 to 28 $\times 10^{-5}$ μc/cc averaging 13 $\times 10^{-5}$ μc. Sixty per cent of the observations in the patients with thyrotoxicosis were above 50 $\times 10^{-5}$ μc, whereas in the euthyroid subjects, 70% were below 20 $\times 10^{-5}$ μc.

There was considerable overlap in the total serum counts (Fig. 2). In the thryotoxic patients the total serum activity ranged from 199 to 659 counts/min./cc and in the euthyroid subjects from 50 to 715 counts/min./cc.

Comment. The tracer technic has been increasingly employed in studies of thyroid function. After an oral dose of I^{131}, urinary excretion accounts for most of the loss from the body;[9-11] the remainder as measured by external counts is mainly in the thyroid gland. A more direct estimation of thyroid function is afforded by the serum level of protein bound radioactive iodine. The higher protein bound I^{131} serum level found in thyrotoxicosis is consistent with increased thyroid activity in these patients. The difference in protein bound radioactive iodine serum levels in euthyroid and thyrotoxic subjects has proved useful diagnostically. Our results indicate, that while the counts/cc of whole serum drawn 24 hours after the oral dose are generally lower in euthyroid subjects than in thyrotoxics, the overlap (Fig. 2) precludes differentiation by the relatively simple determination of total serum activity. This overlap may not be present 72 or 96 hours after a tracer dose and is the subject of further studies.

The factors which influenced our choice of a 24-hour period may be summarized. In rats, Chaikoff, Taurog, and Reinhardt[8] have shown that 24 hours after an injection of I^{131}, approximately 90% of the plasma radioactivity is in protein bound form. Preliminary studies[12] on serum obtained from thyrotoxic patients 1, 3, 6, 24, 48 and 72 hours

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[9] Hamilton, J. G., and Soley, M. H., *Am. J. Phys.*, 1939, **127**, 557.

[10] Hertz, S., Roberts, A., and Salter, W. T., *J. Clin. Invest.*, 1942, **21**, 25.

[11] Keating, R. G., Power, M. H., Berkson, J., Haines, S. F., Trans. Am. Assn. Study of Goiter, 1947, pp. 201-215.

[12] Unpublished data.

pellets intrasplenically, and 7 were reserved as untreated controls. Of a group of 20 mice ovariectomized 47 to 49 days earlier, ten were given progesterone pellets subcutaneously, and 10 were given progesterone pellets intrasplenically.

The pellets were made of crystalline progesterone‡ and weighed 1 to 3 mg. Each animal received a single pellet. The subcutaneous pellets were implanted on the flank by means of a trocar and without anesthesia. The intrasplenic pellets were implanted under sodium amytal-ether anesthesia; splenic hemorrhage was controlled by packing wedges of muscle in the wound.

Four days after implantation of the pellets the mice ovariectomized at the beginning of the shorter period were bled by cardiac puncture. The blood from the members of each of the three subgroups (untreated, with subcutaneous pellets, with intrasplenic pellets) was pooled. Six, 8, 12, 16 and 22 days after implantation of the pellets two mice ovariectomized at the beginning of the longer period and carrying intrasplenic pellets, and 2 carrying subcutaneous pellets, were bled by cardiac puncture. The bloods from mice with the same histories were pooled. Sodium citrate was employed as the anticoagulant. Each pooled sample was centrifuged, and the free and bound fractions of progesterone in the plasma were separated by modification[1] of the method of Szego and Roberts[10] for separation of free and protein-bound estrogen. Briefly, the method involved precipitation of the plasma proteins with 10 volumes of acetone in the cold. The acetone solution contained the free progesterone, and the bound progesterone was freed by partial acid hydrolysis of the proteins. The two fractions were dissolved in sesame oil, and the progesterone content of each was assayed by intra-uterine injection in mice, a method that detects 0.0002 μg.[11]

After bleeding each mouse was autopsied, and the uterus was fixed in Lavdowsky's fluid[12] and prepared for microscopic study.

Findings. The pellets were intact and correctly located. No splenic adhesions were encountered in the animals carrying intrasplenic pellets. The uteri of most of the mice with subcutaneous pellets were of varying shades of yellow and slightly but distinctly enlarged; the uteri of the mice that had carried intrasplenic pellets for 22 days also had this appearance. The uteri of the other treated mice were like those of the untreated animals.

Microscopically, the endometria of the untreated mice showed no evidence of the action of progesterone; the stromal nuclei were shrunken, fusiform, and dense. Similarly, the mice carrying intrasplenic pellets showed no effects of progesterone upon the endometrium. The endometria of all of the mice carrying subcutaneous pellets for eight days or longer showed characteristic progestational changes in the stromal nuclei; they were enlarged, spherical, vesicular, and had distinct nucleoli.[13] When the subcutaneous pellets had been in place only four or six days no change in the stromal nuclei from the castrate condition was evident.

Assays of plasma from the untreated castrates revealed no free progesterone and at most a trace of bound progesterone.

The values for plasma progesterone in the mice carrying pellets are shown in Fig. 1. The animals carrying subcutaneous pellets had plasma levels that increased with the length of time the pellet was in place; indeed, the total progesterone levels (the sum of the free and bound fractions) fluctuated about a straight line when plotted against time. The level of free progesterone attained before an endometrial response was given was roughly 1.0 μg per ml.

The animals with intrasplenic pellets, on the other hand, had consistently low levels of free progesterone, the highest being 0.7 μg per ml. The bound progesterone, however, soon reached a high level in these animals, 2.0 μg per ml by the eighth day after implantation of

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‡ The progesterone was generously supplied by Dr. Erwin Schwenk of the Schering Corporation.

[10] Szego, C. M., and Roberts, S., *Endocrinology*, 1947, **41**, 322.

[11] Hooker, C. W., and Forbes, T. R., *Endocrinology*, 1947, **41**, 158.

[12] Williams, W. L., and Hodge, H. C., *Anat. Rec.*, 1943, **87**, 181.

[13] Hooker, C. W., *Anat. Rec.*, 1945, **93**, 333.

Summary. 1. The serum protein bound radioactive iodine (I^{131}) level was determined 24 hours after the oral administration of 150 microcuries I^{131}. Twenty subjects were studied; 10 were thyrotoxic and 10 euthyroid. The protein bound I^{131} was determined by a modification of the method described by Chaikoff *et al.*[8]

2. The serum protein bound I^{131} in thyrotoxic patients was 38 to 146 × 10^{-5} microcuries/cc averaging 68. In euthyroid subjects, the serum level ranged from 3 to 28 × 10^{-5} microcuries/cc, averaging 13.

3. It would appear that this test may be of diagnostic value as a measure of thyroid function.

17033

Inactivity of Bound Plasma Progesterone.[*]

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Observations have been reported indicating that progesterone in blood is in the plasma and not in the cells and that most of the total progesterone is free while rarely more than 10% is bound to protein or some other substance or substances that render this portion of the progesterone insoluble in acetone and ether.[1] The latter circumstance contrasts with the finding that approximately two-thirds of the estrogen in blood is bound.[2-4] It has been suggested that bound estrogen serves in effect as a reservoir, that the estrogen readily dissociates at the cell membrane, and that the bound fraction is potentially active estrogen.[3] Several observations indicate that a comparable situation does not obtain with respect to bound progesterone. The relative amount of the bound fraction seems too small to serve as a significant source of free progesterone; when introduced directly into the uterus of the mouse the bound fraction had no effect in a test period of 48 hours, presumably sufficient time to permit dissociation if it is to occur; the activity of raw plasma is identical with that of the free progesterone it contains. In short, bound progesterone appeared to be biologically inert.

When progesterone is introduced into the spleen or portal vein and must pass through the liver before reaching the systemic circulation the absorbed progesterone is inactivated in the sense that no effects of the substance are observed in rats,[5] rabbits,[6,7] and mice.[8] If the blood of such animals should contain levels of bound progesterone that would be effective if free, it would constitute further and probably strong evidence for the biological inactivity of the bound fraction. Such an experiment is described here.

Experimental. Young adult mice were used as test animals; the inbred A strain of Strong[9] was chosen to minimize variability. Of a group of 22 animals ovariectomized 13 days earlier, seven were given progesterone pellets subcutaneously, eight were given progesterone ing of an apparently otherwise unaltered steroid compound is a method of inactivation peculiar to progesterone has not been determined.

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[†] Present address: Emory University School of Medicine, Emory University, Ga.

[1] Hooker, C. W., and Forbes, T. R., *Endocrinology*, 1949, **44**, 61.

[2] Rakoff, A. E., Paschkis, K. E., and Cantarow, A., *Am. J. Obst. and Gynec.*, 1943, **46**, 856.

[3] Szego, C. M., and Roberts, S., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 161.

[4] Roberts, S., and Szego, C. M., *Endocrinology*, 1946, **39**, 183.

[5] Selye, H., *J. Pharm. and Exp. Therap.*, 1941, **71**, 236.

[6] Kochakian, C. D., Haskins, A. L., Jr., and Bruce, R. A., *Am. J. Physiol.*, 1944, **142**, 326.

[7] Engel, P., *Endocrinology*, 1946, **38**, 215.

[8] Hooker, C. W., and Li, M. H., unpublished.

[9] Strong, L. C., *J. Hered.*, 1936, **27**, 21.

No explanation can be offered at present for the apparent plateau in bound and total progesterone beginning eight days after implantation of the intrasplenic pellets. Obviously, the rate of absorption may have stabilized or declined, or the rate of removal of bound progesterone from the circulation by excretion or chemical alteration may have increased. Another possibility is that of increasing hepatic inactivation of the progesterone as absorbed by means other than binding.

The apparently linear relation of free and total plasma progesterone to time after the implantation of subcutaneous pellets also raises problems. One of the more interesting of these is the increase in the level of bound progesterone in these animals, possibly suggesting eventual hepatic inactivation of part of the absorbed material by binding. Although it may prove to be of no significance, it is interesting that the maximal level of bound progesterone was almost identical in the two groups of animals.

An interesting fact that emerges is that the minimal physiologically effective plasma level of progesterone with respect to the endometrium appears to be of the order of 1.0 μg per ml of the free fraction. Every animal with this or a higher level exhibited progestational changes in the stromal nuclei. All animals with lower plasma levels showed no endometrial response, irrespective of the level of bound progesterone.

Summary. Pellets of crystalline progesterone implanted into the spleens of ovariectomized mice had no effect upon the endometrium despite the presence of relatively high levels of bound progesterone in the plasma; the highest level of free progesterone in these animals was 0.7 μg per ml. Subcutaneous pellets of progesterone produced characteristic progestational changes in the endometrium when the level of free plasma progesterone exceeded 1.0 μg per ml. Binding appears to be a mechanism of hepatic inactivation of progesterone in the mouse.

17034

Effect of Dialyzed Enterogastrone Upon Twelve-Hour Nocturnal Gastric Secretion in Man.*

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Previous studies[1] have demonstrated occasional decreases in the 12-hour (nocturnal) and 24-hour gastric secretion in man following the intramuscular injection of 1000 to 3000 mg of an enterogastrone concentrate. This effect was variable in degree and temporary in duration. The administration of 400 to 2000 mg did not alter significantly the secretory response of the human stomach to the single standard dose of histamine or to the repeated injection of small amounts of histamine.[2] Insulin-stimulated secretion likewise was not affected, although the response possibly was modified by doses of 2000 or 3000 mg. Ferayorni, Code, and Morlock[3] similarly noted no change in gastric secretion during a double histamine test in 10 human subjects given 200 mg of enterogastrone intramuscularly. In 14 volunteers, quantities up to 400 mg intramuscularly and 18 g orally

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* This study was supported in part by a grant from the Upjohn Co., Kalamazoo, Mich.

[1] Kirsner, J. B., Levin, E., and Palmer, W. L., *Gastroenterology*, 1948, **10**, 256.

[2] Levin, E., Kirsner, J. B., and Palmer, W. L., *Gastroenterology*, 1948, **10**, 274.

[3] Ferayorni, R., Code, C. K., and Morlock, C. G., *Gastroenterology*, 1948, **11**, 730.

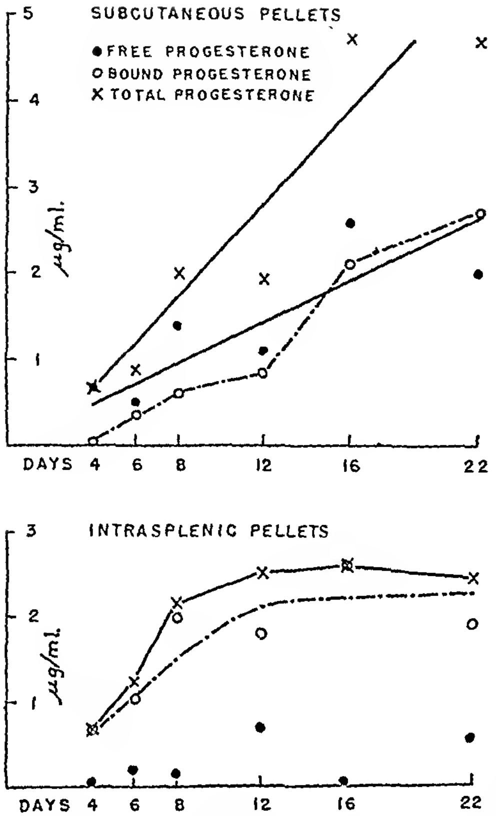

FIG. 1.

Plasma levels of free, bound, and total progesterone in ovariectomized mice with subcutaneous pellets and intrasplenic pellets of progesterone.

the pellets. Here the total plasma progesterone, in contrast to that of the animals with subcutaneous pellets, reached a plateau on the eighth day.

Discussion. All of the observations to date are in agreement in indicating biological inactivity of bound progesterone. The level of this fraction has been consistently low in various ovarian states;[1,14] amounts that would be effective if free have had no effect upon the endometrium when injected directly into the uterus of the mouse;[1] the effectiveness of raw plasma has been the same as that of the free progesterone it contains;[1] in the present experiment circulating levels of bound progesterone that regularly elicited endometrial responses when free were without effect even when present for presumably as long as 16 days.

The identity of the substance to which bound progesterone in the plasma is attached is unknown. It may be protein, as believed for estrogen.[2,3] On the other hand, the method employed for fractionation would probably not distinguish protein-bound material from conjugated material, such as a sulphate or a glucuronide, inasmuch as such conjugants would presumably be precipitated along with the plasma proteins by excess acetone. Whatever its nature, the binding appears to be firm and to dissociate slowly. This is suggested by the lack of response when bound progesterone is injected directly into the uterus and the endometrium is examined 48 hours later, presumably an adequate time for dissociation if it is to occur.[1] No or slow dissociation is further indicated by the present observation that levels of bound progesterone as high as 2.7 μg per ml may be present in the plasma for presumably as long as 16 days without dissociation of enough free progesterone to evoke a recognizable response in the endometrium. Accordingly, binding seems to serve no useful function in the circulation or availability of progesterone.

On the other hand, binding of progesterone is at least one of the methods of inactivating this hormone when it is absorbed in the spleen of the mouse. These observations do not show whether the binding and inactivation occurred in the liver or in the spleen, or indeed in the portal vein. Other studies,[5-7] however, have shown that the liver can inactivate this steroid, and have not revealed appreciable inactivation by the spleen. So far as we are aware, hepatic inactivation of steroids has been found in the past to involve alteration of the steroid compound.[15-20] Whether bind-

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[14] Forbes, T. R., and Hooker, C. W., unpublished.

[15] Heller, C. G., *Endocrinology*, 1940, **26**, 619.

[16] Schiller, J., *Endocrinology*, 1945, **36**, 7.

[17] Pearlman, W. H., Paschkis, K. E., Rakoff, A. E., Cantarow, A., Walkling, A. A., and Hansen, L. E., *Endocrinology*, 1945, **36**, 284.

[18] Samuels, L. T., McCaulay, C., and Sellers, D. M., *J. Biol. Chem.*, 1947, **168**, 477.

[19] Levy, H., *Arch. Biochem.*, 1947, **14**, 325.

[20] DeMeio, R. H., Rakoff, A. E., Cantarow, A., and Paschkis, K. E., *Endocrinology*, 1948, **43**, 97.

ing of an apparently otherwise unaltered steroid compound is a method of inactivation peculiar to progesterone has not been determined.

No explanation can be offered at present for the apparent plateau in bound and total progesterone beginning eight days after implantation of the intrasplenic pellets. Obviously, the rate of absorption may have stabilized or declined, or the rate of removal of bound progesterone from the circulation by excretion or chemical alteration may have increased. Another possibility is that of increasing hepatic inactivation of the progesterone as absorbed by means other than binding.

The apparently linear relation of free and total plasma progesterone to time after the implantation of subcutaneous pellets also raises problems. One of the more interesting of these is the increase in the level of bound progesterone in these animals, possibly suggesting eventual hepatic inactivation of part of the absorbed material by binding. Although it may prove to be of no significance, it is interesting that the maximal level of bound progesterone was almost identical in the two groups of animals.

An interesting fact that emerges is that the minimal physiologically effective plasma level of progesterone with respect to the endometrium appears to be of the order of 1.0 μg per ml of the free fraction. Every animal with this or a higher level exhibited progestational changes in the stromal nuclei. All animals with lower plasma levels showed no endometrial response, irrespective of the level of bound progesterone.

Summary. Pellets of crystalline progesterone implanted into the spleens of ovariectomized mice had no effect upon the endometrium despite the presence of relatively high levels of bound progesterone in the plasma; the highest level of free progesterone in these animals was 0.7 μg per ml. Subcutaneous pellets of progesterone produced characteristic progestational changes in the endometrium when the level of free plasma progesterone exceeded 1.0 μg per ml. Binding appears to be a mechanism of hepatic inactivation of progesterone in the mouse.

17034

Effect of Dialyzed Enterogastrone Upon Twelve-Hour Nocturnal Gastric Secretion in Man.*

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Previous studies[1] have demonstrated occasional decreases in the 12-hour (nocturnal) and 24-hour gastric secretion in man following the intramuscular injection of 1000 to 3000 mg of an enterogastrone concentrate. This effect was variable in degree and temporary in duration. The administration of 400 to 2000 mg did not alter significantly the secretory response of the human stomach to the single standard dose of histamine or to the repeated injection of small amounts of histamine.[2] Insulin-stimulated secretion likewise was not affected, although the response possibly was modified by doses of 2000 or 3000 mg. Ferayorni, Code, and Morlock[3] similarly noted no change in gastric secretion during a double histamine test in 10 human subjects given 200 mg of enterogastrone intramuscularly. In 14 volunteers, quantities up to 400 mg intramuscularly and 18 g orally

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* This study was supported in part by a grant from the Upjohn Co., Kalamazoo, Mich.

[1] Kirsner, J. B., Levin, E., and Palmer, W. L., *Gastroenterology*, 1948, **10**, 256.

[2] Levin, E., Kirsner, J. B., and Palmer, W. L., *Gastroenterology*, 1948, **10**, 274.

[3] Ferayorni, R., Code, C. K., and Morlock, C. G., *Gastroenterology*, 1948, **11**, 730.

did not diminish the response to a modified Ewald test meal. Pollard and his associates[4] administered 8 to 16 g of the concentrate by mouth daily to 12 patients with peptic ulcer for as long as 11 months; 16 individuals received intramuscular injections of 200 mg daily for intervals up to 9 months. There were no changes in the volume of fasting gastric secretion, output of hydrochloric acid, concentration of pepsin or gastric motility.

The enterogastrone utilized in the initial experiments, although less crude than earlier products, consisted of a mixture of proteins of varying molecular size; its chemical composition is not known, and its physiologic characteristics are obscure. The most recent preparation of enterogastrone[†] is described[5] as free of inactive protein by dialysis through a cellophane membrane. Inhibition of gastric secretion in the rat and the dog approximated twice the degree obtained with the standard preparation. Neither this product nor other samples of enterogastrone have manifested anti-secretory activity in the guinea pig. Tests for toxicity are reported as satisfactory at 500 mg/kg and for vasodepressor activity as meeting present requirements.

The present study was undertaken to determine the effect of this concentrate upon the nocturnal gastric secretion of patients with peptic ulcer.

Method of study. The procedure was the same as that described in previous papers.[6-8] Ten male patients, 9 with active duodenal ulcer and one with a benign gastric ulcer, were studied. Constant suction of the gastric content was maintained by a Gomco aspirator. The volume and free acidity of each hourly collection were measured and the output of hydrochloric acid in milligrams calculated from these data. In 8 patients, the stomach was aspirated continuously for 60 hours, beginning at 9:30 P.M. After 48 hours had elapsed the enterogastrone was administered at 9:30 P.M. in single total doses of 200 to 5000 mg. The initial 12 hours (9:30 P.M. to 9:30 A.M.) constituted the control nocturnal period; the 12 hours (9:30 P.M. to 9:30 A.M.) after the injection of the concentrate represented the test period. In one case the control interval extended during the night and morning (11:30 P.M. to 11:30 A.M.); 2000 mg of enterogastrone then were given and the hourly secretion measured for the subsequent 12 hours (11:30 A.M. to 11:30 P.M.). A similar procedure was followed in the tenth patient; the control period was of 10 hours duration (11:30 P.M. to 9:30 A.M.); 5000 mg of enterogastrone then were administered and the gastric aspirations continued for the succeeding 10 hours (9:30 A.M. to 7:30 P.M.).

The patients were not given food or liquid by mouth. Normal electrolyte and fluid balances were maintained by the intravenous administration of 5% glucose in isotonic saline solution. The enterogastrone was injected intramuscularly into the glutei in a single dose. Pain developed locally almost at once, and usually was so intense as to prevent sleep. The body temperature remained normal in 7 cases; a slight elevation to 37.6 or 37.8°C occurred in 3 individuals (A.H., W.W., and C.M.) during the final one or two hours of the test period. No other untoward effects were observed.

Results. The data are recorded in Table I. The 12-hour gastric secretion was not reduced significantly in the four patients receiving 200 to 2000 mg of enterogastrone. The output of acid was decreased in each of the 6 individuals given 5000 mg of the preparation. The inhibition was slight in one and moderate or pronounced in 5. Depression of gastric secretion became apparent immediately in one case and within 2 or 3 hours in the remaining 5 patients. Anacidity for periods of 5 to 9

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[4] (a) Pollard, H. M., Block, M., and Bachrach, W. H., *Proc. Central Society Clinical Research*, 1946, **19**, 34. (b) Pollard, H. M., Block, M., Bachrach, W. H., and Mason, J., *Arch. Surg.*, 1948, **56**, 372.

[5] Hailman, H. F., and Visscher, F., personal communication.

[†] Supplied by Dr. H. F. Hailman, Upjohn Co., Kalamazoo, Mich.

[6] Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Arch. Surg.*, 1948, **56**, 345.

[7] Kirsner, J. B., Levin, E., and Palmer, W. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 108.

[8] Levin, E., Kirsner, J. B., and Palmer, W. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 153.

TABLE I.

Effect of Dialyzed Enterogastrone on 12-Hour Nocturnal Gastric Secretion of 10 Patients with Peptic Ulcer.

Case	Control			Amt *enterogastrone* intramusc. (mg)	After enterogastrone			Comment
	Vol. (cc)	*Free HCl* (Cl. units)	*Total output* HCl (mg)		*Vol.* (cc)	*Free HCl* (Cl. units)	*Total output* HCl (mg)	
♂—27 D.U.	976	79	2815	200	1114	77	3138	No effect
♂—42 D.U.	1033	49	1829	500	1047	38	1449	No significant effect
♂—39 D.U.	923	81	2719	1000	1130	85	3492	No effect
♂—51 D.U.	814	73	2487	2000	1054	70	3088	No effect
♂—57 D.U.	1056	87	3340	5000	1099	56	2646	Slight decr. 2 hr after inj.; reduced acid 2 hr., anacidity 2 hr
♂—34 D.U.	822	74	2218	5000	762	39	1071	Marked decr.; anacidity 3 hr after inj., continued for 7 hr
♂—72 D.U.	1647	48	2974	5000	1151	2	107	Marked decr. apparent immed.; anacidity 11 of 12 hr
♂—54 D.U.	1494	95	4874	5000	725	22	1251	Marked decr. after 3 hr.; anacidity 7 to 12 hr
♂—64 D.U.	1684	101	6220	5000	703	31	1501	Marked decr. after 3 hr.; anacidity 5 hr
♂—43 G.U.	519	12 (10 hr)	260	5000	332	6 (10 hr)	108	Moderate decr.; anacidity after 2 hr

consecutive hours was noted in 5 instances; in one case there was no free acid in 11 of the 12 specimens. However, hydrochloric acid usually reappeared towards the conclusion of the nocturnal period. Free acid had been present continuously during the control periods in the 9 patients with duodenal ulcer. The inhibition of secretion consisted usually of a pronounced decrease in the concentration of acid. However, the volume of gastric content also diminished markedly in 3 patients.

Comment. The present data indicate that the fasting gastric secretion may decrease greatly, albeit temporarily, following the intramuscular administration of 5000 mg of a dialyzed preparation of enterogastrone. This finding is in contrast to a single previous experiment in which gastric secretion was unaffected by the injection of 5000 mg of an apparently less concentrated product. Although the injections produced severe pain immediately, the decrease in acid usually did not become apparent until two or three hours had elapsed. As in preceding studies, the effect consisted chiefly of a lowered concentration of acid; the volume of secretion also diminished significantly in three of the present series. The mechanism of this reduction and its significance remain obscure. It may be noted that the intramuscular administration of a non-specific protein, sterile lactalbumin, did not reduce the nocturnal gastric secretion in 10 patients with peptic ulcer; indeed, an increased output of acid was noted in 6.[9] The inhibition is not attributable to an elevation in body temperature, since the temperature increased in only three patients and then very slightly; the reduction in acid in one of this group was slight. The temporary duration of the inhibition, the tremendous quantities required, the accompanying severe pain, and the uncertainty as to the nature of the material and the mechanisms involved do not warrant further clinical trial of the present dialyzed preparation of enterogastrone. Nevertheless, the data would appear to justify the continued search for a much more effective and safely administered product, whose chemical nature and physiological behavior may be studied more precisely.

Conclusions. 1. The output of hydrochloric acid in the 12-hour nocturnal gastric secretion of 4 patients with peptic ulcer was unchanged following the intramuscular administration of 200 to 2000 mg of a dialyzed preparation of enterogastrone. 2. Gastric secretion was reduced slightly in one and markedly in five of six patients with peptic ulcer, given 5000 mg. The transitory inhibition consisted chiefly of a decrease in the concentration of acid and, to a lesser extent, of the volume of secretion.

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[9] Kirsner, J. B., Levin, E., and Palmer, W. L., Proc. Soc. Exp. Biol. and Med., 1948, 68, 90.

17035

Thiosemicarbazide: A New Toxic Derivative of Thiourea.*

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Previous papers from this laboratory[1-4] have described the acute toxicity of thiourea and a number of its singly substituted derivatives, notably alpha-naphthyl thiourea (ANTU), which has proved to be an effective rodenticide for the field control of Norway rats.[1] The subject of the present paper is thiosemicarbazide ($NH_2NHCSNH_2$), in

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* Carried out under a contract between the Medical Division, Chemical Corps, U. S. Army, and The Johns Hopkins University. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the author.

TABLE I.
Acute Toxicity of Thiosemicarbazide.
(Adult animals, except as noted).

Species	No. used	Administered*	LD_{50} ± S.E.† mg/kg B.W.
Wild Norway rat *(Rattus norvegicus)*	Adult 56	s.t.	13 ± 2.1
	Young 21	”	19 ± 1.3
Laboratory rat *(Rattus norvegicus)*	Strain I 49	”	11 ± 2.0
	Strain II 44	”	18 ± 2.0
Alexandrine rat *(Rattus rattus)*	26	”	23 ± 1.4
Cotton rat *(Sigmidon hispidus hispidus)*	31	”	16 ± 2.2
Guinea pig *(Cavia cobaya)*	37	i.p.	24 ± 2.0
Dog *(Canis familiaris)*	6	s.t.	10 (5-15)
Cat *(Felix libyca domestica)*	4	”	20 (15-?)

* s.t. = by stomach tube; i.p. = by intraperitoneal injection. The vehicle was, in every instance, a 10% solution of gum acacia in water.

† LD_{50}'s and their standard errors were obtained by the method of Litchfield and Fertig.[7] Whenever data were not sufficiently extensive to justify statistical treatment, an estimate of the LD_{50} is followed by the range between the highest dose observed to kill none and the lowest dose killing all.

which an amino group replaces the naphthyl radical of ANTU. This compound differs in several respects from the other toxic thioureas, owing presumably to the fact that it is a derivative of hydrazine (NH_2NH_2) as well as of thiourea (NH_2CSNH_2).

The rapid death of white rats following administration of thiosemicarbazide was first observed by Dr. Emanuel Waletzky of the American Cyanamid Company; Dr. R. O. Roblin of that company then sent a sample to this laboratory for testing on wild Norway rats as a possible new rodenticide. Observations that this substance was toxic have also been made by Astwood[5] and by Jensen and Kjerulf-Jensen[6] in the course of their studies on the chronic feeding of various chemicals to determine their goiterogenic activity.

Acute Toxicity. Table I summarizes the data obtained with the 6 different species of animals available for assay. The thiosemicarbazide was administered either by stomach tube or by intraperitoneal injection, in water containing 10% gum acacia, according to the technic previously used for assaying ANTU.[3] Two strains of laboratory Norway rats were used to compare with the wild Norways, because previous work had shown that very marked differences in susceptibility to thiourea existed between domestic rats from different colonies.[2] The rats designated as strain I came from our own colony, which shows uniformly high susceptibility to thiourea poisoning; while those of strain II came from a colony of Wistar rats maintained at the Army Chemical Center, Edgewood, Maryland. The wild Norway rats were freshly trapped speci-

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[1] Richter, C. P., *J. A. M. A.*, 1945, **129**, 927.

[2] Dieke, S. H., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1945, **83**, 195.

[3] Dieke, S. H., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 22.

[4] Dieke, S. H., Allen, G. S., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1947, **90**, 260.

[5] Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, **78**, 79.

[6] Jensen, K. A., and Kjerulf-Jensen, K., *Acta Pharmacol.*, 1945, **1**, 280.

[7] Litchfield, J. T., Jr., and Fertig, J. W., *Bull. Johns Hopk. Hosp.*, 1941, **69**, 276.

mens from the alleys of Baltimore. The Alexandrine and cotton rats came from our own colonies. All the rats were fed purina fox chow while in this laboratory.

Table I shows that all the acute median lethal doses obtained fell between 10 and 25 mg/kg. For comparison, the equivalent values for ANTU ranged from 2.5 mg/kg for domestic Norway rats up to about 500 mg/kg for cats (and over 4 grams/kg for monkeys and chickens).[3] This indicates that thiosemicarbazide does not share with ANTU a specific toxicity to Norway rats, but is more widely toxic to warm blooded animals in general.

One monkey (*Macaca mulatta*) was also available for testing the toxicity of thiosemicarbazide; it received 200 mg/kg by stomach tube and died within 24 hours.

The acute toxicity of thiosemicarbazide to young Norway rats was tested, since previous work showed that young rats were 6 or 7 times less susceptible to ANTU than were adults.[3] Thiosemicarbazide was given to 21 young wild Norway rats weighing between 36 g and 110 g and all sexually immature. The LD50 obtained with this limited number of rats (See Table I) was less than twice that found for adults, which indicates that compared to ANTU, the response to thiosemicarbazide is substantially less variable with age. This is borne out by the results of acceptance tests, described below.

Both male and female animals of every species were used at most assay levels. A separation of the data gave no indication of any difference in response that could be attributed to sex.

In all the animals included in Table I, with the significant exception of some laboratory Norway rats, the effects noted after poisoning were very similar. Within an hour after dosing the animals appeared to be in a highly excited and apprehensive condition. The rats and guinea pigs frequently squealed, the cats miaowed, and the dogs barked or growled. Violent convulsions then started, often terminating within a minute or two with the teeth bared and the limbs stiffly extended, only to begin again within a matter of minutes. Intense salivation was observed in all animals receiving fatal doses, and vomiting in the dogs, cats, and monkey. Death or what appeared to be complete recovery occurred within 6 hours, except for the monkey which survived for almost a day.

On the other hand, laboratory Norway rats of both strains often reacted differently to thiosemicarbazide, especially after low but nevertheless fatal doses. Those from our colony (Strain I) frequently had only mild convulsions or none at all, and deaths usually did not occur until after 18 to 24 hours. At autopsy these rats showed pulmonary edema accompanied by pleural effusion (as found in typical ANTU or thiourea poisoning). The other laboratory rats (Strain II) usually died in convulsions, but some of these also had none and died overnight with pulmonary edema.[†] In none of the other animals receiving thiosemicarbazide (including the monkey) was any pleural effusion found, nor was any significant amount of pulmonary edema evident either grossly or in histological sections of the lungs. This interesting finding is reminiscent of results previously reported,[2] in which poisoning of rats from our colony with the parent compound thiourea produced pulmonary edema, while equivalent doses had no effect on the lungs of wild Norway rats.

Acceptability. The voluntary consumption of thiosemicarbazide appears to be good, on the basis of tests made with individually caged rats. A concentration of 0.5% in either water solution or in a bait of yellow cornmeal killed 18 of 19 wild Norways, all of 8 Alexandrines, and 7 of 8 cotton rats. At 1% in bait all of 12 wild Norways died, and at 2% 7 out of 8. With 20 laboratory Norways (Strain I), divided into 3 groups, all of 8 died after drinking a concentration of 0.25% in water, and 4 of 8 at a 0.1% concentration, while none of 4 died from drinking a 0.05% solution. No hesitation to drink these solutions was observed, despite the fact that thiosemicarbazide has a bitter taste to humans and most probably also to rats.[8]

[†] Dr. Waletzky states (private communication) that rats from his colony, originating from Carworth Farms for the most part, exhibited violent convulsions and died in 1 to 3 hours. These rats would thus seem to resemble wild Norways more than the rats from our colony.

Of the 39 wild Norway rats included in this test, 29 were immature, weighing between 54 and 130 g. All but one of these young rats died after ingesting doses as low as 15 mg/kg, which indicates that this poison should be effective against young as well as adult wild Norway rats.

Tolerance. Thirty-six adult wild Norway rats and 6 adult laboratory Norway rats (Strain I) were used to investigate the possible development of tolerance to thiosemicarbazide. The wild Norways all received an initial dose of 7.5 mg/kg by stomach tube (approximately ½ an LD50 dose). Seven received 4 subsequent doses of 7.5, 7.5, 20, and 50 mg/kg, also by stomach tube, on alternate days thereafter. Convulsions were observed in 2 of these 7 rats following the 20 mg/kg dose, but all survived. All died within 3 hours after receiving 50 mg/kg, in violent convulsions.

Fourteen other wild Norways received 30 mg/kg on their second dose: 3 out of 3 died when the interval between doses was 14 days, 4 out of 4 when 3 days had elapsed, but only 3 out of 7 at 2 days after the initial poisoning.

When the second dosing was accomplished by offering poisoned water (0.25%) for voluntary consumption, 4 out of 4 died when the interval was one day, and at 2 days 7 out of 10. No wild Norway survived a dose higher than 60 mg/kg at any time. It would seem, then, that a tolerance to 4 or 5 median lethal doses can develop in wild Norway rats, and that this tolerance is most marked about 2 days after a sublethal dose. This is not serious from a practical standpoint, as the consumption of 5 cc of poisoned water (at 0.25%) or a few grams of poisoned food would provide ample thiosemicarbazide to offset such a tolerance, even in large sized rats.

In contrast to the above results, somewhat larger tolerances were established in the laboratory rats (Strain I). These rats received by stomach tube an initial dose of 5 mg/kg (or about ½ an LD50) and subsequent doses on alternate days of 5, 5, 20, 50, 100, 150, 200, and 300 mg/kg. One rat died (with pulmonary edema) from the initial dose, but no other fatalities occurred until 2 of the remaining 5 succumbed to 100 mg/kg. Two others died following 200 mg/kg and the last after 300 mg/kg. Thus 1 rat developed a tolerance to about 20 times the LD50 dose, on a regimen which with ANTU produces tolerances of 80 times with little difficulty, even in wild Norway rats.[1]

Antidote. The copious salivation found in animals poisoned with thiosemicarbazide suggested that atropine might be an antagonist. Preliminary experiments with 8 rats, one dog and one cat were unsuccessful; in fact, although the salivation was suppressed, the animals appeared to die rather sooner when treated with amounts of atropine which were not lethal to controls than when they received the thiosemicarbazide alone.

On the other hand the administration of 20 mg/kg of sodium pentobarbital at the onset of the first violent convulsion (about one hour after poisoning) followed by another similar amount when necessary, was effective in preventing the death of 2 dogs and one cat, each of which had received 5 times an LD50 dose. The convulsions were not entirely prevented by this amount of pentobarbital, but their intensity was lessened. Six hours after poisoning all 3 animals looked well although a little groggy; the next day recovery appeared to be complete.

The treatment of 16 laboratory rats (Strain II) with 40 mg/kg of sodium pentobarbital (and subsequent booster doses when necessary), at the onset of convulsions following either 3 or 6 LD_{50}'s of thiosemicarbazide produced some interesting results. Whereas the controls all died within 3 hours, none of the rats kept unconscious by the barbiturate died within the first 5 hours. Only 3 out of 8 survived the lower dose, however, and only one of the 8 receiving the higher dose: the rest died overnight with pulmonary edema and pleural effusion. It would therefore appear that pentobarbital is an antidote for those species in which thiosemicarbazide has only a convulsant action, but not when it also has an ANTU-like action on the lungs. Since 2 dogs survived relatively large doses when treated with this barbiturate, it may be that, unlike ANTU, thiosemicarbazide would not

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[8] Richter, C. P., in press.

cause pulmonary edema in this species as it does in the laboratory Norway rat.

Comparison with other poisons. Some of the effects produced by thiosemicarbazide indicate the relationship of this compound to hydrazine. According to Fränkel,[9] hydrazine causes excitement and occasionally convulsions in higher animals, but does not damage erythrocytes as does phenyl hydrazine. O. Loew[10] reports convulsions and rapid death in guinea pigs after fatal doses of hydrazine. Underhill and Karelitz,[11] who studied the anemia caused in dogs by hydrazine sulfate, observed more or less constant salivation, vomiting and diarrhea; they mention no convulsions, however, nor does Bodansky[12] whose dog also vomited and salivated for several days.

To check the relative effects of thiosemicarbazide and the two compounds from which it derives, 14 laboratory rats (Strain II) were given thiourea and 14 hydrazine (as the sulfate), at the same time and in the same way that the LD50 of thiosemicarbazide was determined. The acute LD50 for thiourea fell at about 20 mg/kg, and that for hydrazine sulfate at about 450 mg/kg.[‡] None of the rats receiving either thiourea or hydrazine sulfate had convulsions; the main autopsy findings in those succumbing to thiourea were pulmonary edema and pleural effusion, while hydrazine sulfate caused increased salivation and a grossly evident liver damage. A chocolate brown blood, as found after poisoning with phenyl hydrazine, was not observed.

The differences between thiosemicarbazide and the other toxic thioureas previously studied are several. Thiosemicarbazide has a strong convulsant action, which ANTU and other similar thioureas do not share, and appears to be quite generally toxic to a number of species of animals, rather than almost specifically toxic to Norway rats. It is not markedly less toxic to young than to adult Norway rats, and sublethal doses do not produce the high tolerances characteristic of ANTU. Furthermore, pentobarbital gives promise of being an adequate antidote, at least for those species not affected by the thiourea moiety of the molecule.

From a practical standpoint, thiosemicarbazide might have a wider applicability than ANTU because it dissolves in water and so can be used as a water poison as well as in bait. It can also presumably be used against other rodents than wild Norway rats; an evaluation of the success of such use must await field trials. On the other hand it is very probably toxic to man as well as to other animals and will therefore require far more caution in use. It acts very quickly and apart from a bitter taste, similar to that of phenyl thiourea and probably also subject to genetically controlled "taste blindness",[13] carries with it no warning. The existence of an antidote under these circumstances is comforting but may not be of much practical value.

Compared to other universal poisons such as sodium fluoroacetate (1080), the value of thiosemicarbazide would seem to lie in its lower toxicity, which under some circumstances might be an advantage, since it would lessen the hazard to larger animals, including man. The consumption of a few grams of bait is sufficient to kill rats, and since the animals feel unwell within a short time after poisoning they rarely take more than that. No individual rat in our tests has voluntarily consumed sufficient poison to kill a (much larger) dog or cat by secondary poisoning, assuming no loss of toxicity of the substance while in the rat's body.

Thiosemicarbazide is a relatively stable compound under ordinary conditions of light-ing, humidity and temperature. It melts at 180°C with decomposition, but below that temperature it appears not to decompose spontaneously. It is soluble in cold water to about 1 or 2% and in hot water to about 10%. It is also soluble in alcohol.§

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[9] Fränkel, S., *Die Arzneimittel-Synthese*, 6th ed., Berlin, 1927, pp. 84-85.

[10] Loew, O., *Ber. d. Deut. Chem. Ges.*, 1890, **23**, 3203.

[11] Underhill, F. P., and Karelitz, S., Jr., *J. Biol. Chem.*, 1923, **58**, 147.

[12] Bodansky, M., *J. Pharm. and Exp. Therap.*, 1924, **23**, 127.

[‡] This last finding confirms a previous report[4] as to the relatively low toxicity of hydrazine sulfate to laboratory rats, and also agrees well with results obtained with a few wild Norway rats, for which strain the LD_{50} probably falls between 250 and 500 mg/kg.

[13] Blakeslee, A. F., and Fox, A. L., *J. Hered.*, 1932, **23**, 97.

Summary. Thiosemicarbazide, a derivative of both thiourea and hydrazine, has proved to differ from other toxic thioureas previously studied. Instead of producing fatal pulmonary edema in a limited number of animal species and not harming others at equivalent dose levels, thiosemicarbazide caused convulsions and death within 1 to 3 hours in the 6 species tested, when given in amounts ranging from 10 to 30 mg/kg. In some individual laboratory rats, however, including those in which the convulsions had been suppressed by administration of a barbiturate, death was delayed and accompanied by the development of pulmonary edema. Thiosemicarbazide may have promise as a practical rodenticide, because of its general toxicity to rats of several species and because it appears to be readily accepted in lethal amounts when offered to rats in either water solution or bait.

It is a pleasure to acknowledge the kindness of Dr. Wayland G. Hayes of the Communicable Disease Center, U. S. Public Health Service, Savannah, Georgia, who provided the nucleus of our Alexandrine rat colony, and of Dr. R. O. Roblin, American Cyanamid Company, who has helped in many ways throughout the course of this study.

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§ The analytical data on thiosemicarbazide given in this paragraph were very kindly provided by Dr. J. R. Vaughan of the American Cyanamid Company.

17036

Estimation of Dicumarol, 3, 3^1-Methylenebis (4-Hydroxycoumarin) in Biological Fluids.*

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Although the physiological activity of dicumarol has been assayed by its effect on the prothrombin time, no chemical method for its estimation has been available. A rapid chemical method would make possible a study of certain problems concerning dicumarol about which little is known. These are its physiological disposition in man, its relationship to vitamin K in the clotting mechanism, and its formation in spoiled sweet clover.

A chemical method is described below which involves isolation of the drug from the body by extraction into heptane. The drug is returned to alkali and measured spectrophotometrically at 315 mμ where the drug exhibits a pronounced peak (Fig. 1).

Reagents. 1. Standard solution of dicumarol 100 mg per liter. 100 mg of dicumarol are dissolved in 1 liter of 0.1 N NaOH. This solution is stable for at least one month when stored in the refrigerator.

2. 3 N HCl.

3. Heptane. (Paragon Testing Company). A technical grade of heptane is purified by successive washings with 1 N NaOH and 1 N HCl followed by 2 washings with water.

4. 2.5 N NaOH.

Procedure. Add 1 to 3 ml of plasma or urine (sample containing up to 50 γ of dicumarol) and 0.5 ml of 3 N HCl to 20 ml of heptane in a 60 ml glass-stoppered bottle. Adjust the aqueous volume to about 3.5 ml if necessary by the addition of water. Shake for 30 minutes on a shaking apparatus and

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* This work was supported by a grant from the Institute for the Study of Analgesic and Sedative Drugs.

cause pulmonary edema in this species as it does in the laboratory Norway rat.

Comparison with other poisons. Some of the effects produced by thiosemicarbazide indicate the relationship of this compound to hydrazine. According to Fränkel,[9] hydrazine causes excitement and occasionally convulsions in higher animals, but does not damage erythrocytes as does phenyl hydrazine. O. Loew[10] reports convulsions and rapid death in guinea pigs after fatal doses of hydrazine. Underhill and Karelitz,[11] who studied the anemia caused in dogs by hydrazine sulfate, observed more or less constant salivation, vomiting and diarrhea; they mention no convulsions, however, nor does Bodansky[12] whose dog also vomited and salivated for several days.

To check the relative effects of thiosemicarbazide and the two compounds from which it derives, 14 laboratory rats (Strain II) were given thiourea and 14 hydrazine (as the sulfate), at the same time and in the same way that the LD50 of thiosemicarbazide was determined. The acute LD50 for thiourea fell at about 20 mg/kg, and that for hydrazine sulfate at about 450 mg/kg.[‡] None of the rats receiving either thiourea or hydrazine sulfate had convulsions; the main autopsy findings in those succumbing to thiourea were pulmonary edema and pleural effusion, while hydrazine sulfate caused increased salivation and a grossly evident liver damage. A chocolate brown blood, as found after poisoning with phenyl hydrazine, was not observed.

The differences between thiosemicarbazide and the other toxic thioureas previously studied are several. *Thiosemicarbazide* has a strong convulsant action, which ANTU and other similar thioureas do not share, and appears to be quite generally toxic to a number of species of animals, rather than almost specifically toxic to Norway rats. It is not markedly less toxic to young than to adult Norway rats, and sublethal doses do not produce the high tolerances characteristic of ANTU. Furthermore, pentobarbital gives promise of being an adequate antidote, at least for those species not affected by the thiourea moiety of the molecule.

From a practical standpoint, thiosemicarbazide might have a wider applicability than ANTU because it dissolves in water and so can be used as a water poison as well as in bait. *It can also presumably be used against* other rodents than wild Norway rats: an evaluation of the success of such use must await field trials. On the other hand it is very probably toxic to man as well as to other animals and will therefore require far more caution in use. It acts very quickly and apart from a bitter taste, similar to that of phenyl thiourea and probably also subject to genetically controlled "taste blindness",[13] carries with it no warning. The existence of an antidote under these circumstances is comforting but may not be of much practical value.

Compared to other universal poisons such as sodium fluoroacetate (1080), the value of thiosemicarbazide would seem to lie in its lower toxicity, which under some circumstances might be an advantage, since it would lessen the hazard to larger animals, including man. The consumption of a few grams of bait is sufficient to kill rats, and since the animals feel unwell within a short time after poisoning they rarely take more than that. No individual rat in our tests has voluntarily consumed sufficient poison to kill a (much larger) dog or cat by secondary poisoning, assuming no loss of toxicity of the substance while in the rat's body.

Thiosemicarbazide is a relatively stable compound under ordinary conditions of light-dose. The distributions of dicumarol between heptane and water at various pH values were compared with those of the apparent compound extracted from plasma. The results showed that within experimental error the apparent and authentic compound had the same solubility characteristics and were, therefore, presumably the same compound (Table I).

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[9] Fränkel, S., *Die Arzneimittel-Synthese*, 6th ed., Berlin, 1927, pp. 84-85.

[10] Loew, O., *Ber. d. Deut. Chem. Ges.*, 1890, **23**, 3203.

[11] Underhill, F. P., and Karelitz, S., Jr., *J. Biol. Chem.*, 1923, **58**, 147.

[12] Bodansky, M., *J. Pharm. and Exp. Therap.*, 1924, **23**, 127.

[‡] This last finding confirms a previous report[4] as to the relatively low toxicity of hydrazine sulfate to laboratory rats, and also agrees well with results obtained with a few wild Norway rats, for which strain the LD$_{50}$ probably falls between 250 and 500 mg/kg.

[13] Blakeslee, A. F., and Fox, A. L., *J. Hered.*, 1932, **23**, 97.

Basic organic drugs do not interfere in the procedure for dicumarol since they are not extracted at an acid pH. The following acidic or neutral drugs were tested for their interference in the procedure for dicumarol: acetanilide, phenacetin, antipyrine, phenobarbital, sulfanilamide, sulfadiazine, sulfathiazole, penicillin, vitamin K, nembutal, pentothal, and salicylic acid. Only pentothal and salicylates interfered in the procedure and consequently these substances should not be present when analyses for dicumarol are being made.

Summary. A simple and sensitive spectrophotometric method for the estimation of dicumarol in plasma and urine is described. Dicumarol is isolated from the biological material by extraction into heptane. The drug is returned to alkali and measured spectrophotometrically at 315 mμ. The method is specific in that it does not include metabolic products of the drug.

17037 P

Seasonal Variations in the Choline Content of Human Serum.

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In the literature there are but few reports of studies on serum choline content (Guggenheim and Loffler,[1] Sieburg and Patzschke,[2] Luecke and Pearson,[3] and Schlegel[4]).

It appears from these studies that the concentration of choline in human serum ranges from about 0.2 to 2 mg %. As far as has been ascertained, no records are available of studies presenting evidence of seasonal variations in serum choline content.

The present study comprises 142 duplicate determinations of serums obtained from both men and women in the period from May 1, 1947 to May 1, 1948. Determinations of choline were made according to the method of Abdon and Ljungdahl-Ostberg[5] by converting choline into acetylcholine, which physiologically is about 100,000 times as active as choline. Its action was determined

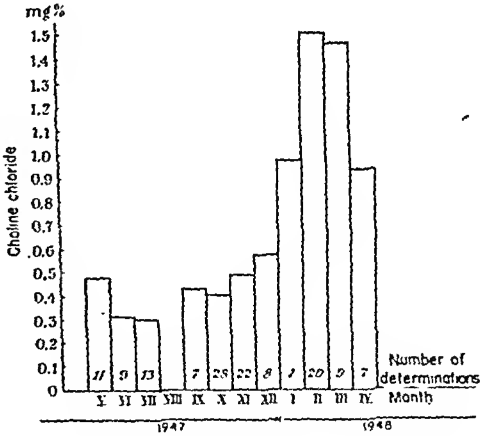

FIG. 1.

The average serum choline content during the different months of the year.

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[1] Guggenheim, M., and Loffler, W., *Biochem. Z.*, 1916, **74**, 303.

[2] Sieburg and Patzschke, *Z. d. ges. exp. Med.*, 1923, **36**, 324.

[3] Luecke, R. W., and Pearson, P. B., *J. Biol. Chem.*, 1944, **153**, 259.

[4] Schlegel, J. U., Variationer i Serumcholinindholdet hos Mennesker, Copenhagen 1948 (Thesis).

[5] Abdon, N. O., and Ljungdahl-Ostberg, K., *Acta Physiol. Scandinav.*, 1944, **8**, 103.

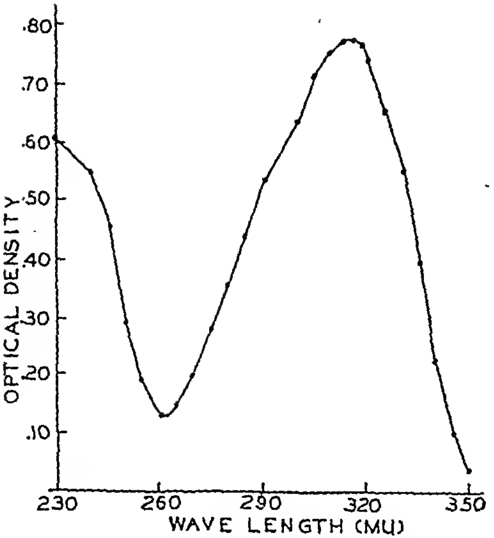

FIG. 1.

The absorption spectrum of dicumarol in 2.5 N NaOH. The concentration of the compound was 10 γ per ml. Cell thickness = 1 cm.

then centrifuge the bottle. Transfer 15 ml of the heptane phase to a 60 ml glass-stoppered bottle containing 4 ml of 2.5 N NaOH. Shake for 5 minutes. Transfer the contents to a test tube and centrifuge for 5 minutes. Remove the organic phase by aspiration with a fine-tipped pipette. Transfer about 3 ml of the aqueous phase to a quartz cuvette and determine the optical density in a spectrophotometer (Beckman) with the instrument set at the wave-length 315 mμ. A reagent blank with water substituted for plasma is run through the same procedure. This blank is used for the zero setting and should have an optical density not greater than 0.005 when 2.5 N NaOH is used for the zero setting.

Standards. The distribution of dicumarol in a heptane-acidified water system is such that at room temperature with volumes of 20 and 3.5 ml respectively, about 95% of dicumarol is in the organic phase. Standards are prepared by handling known amounts of dicumarol as in the procedure described above. The optical densities were found to be proportional to concentration. An optical density of about 0.130 is obtained in a Beckman spectrophotometer when 10 γ of dicumarol are run through the above procedure.

Results. Recoveries of dicumarol added to plasma in amounts of 5 to 50 γ were quite satisfactory (100 ± 3%). The sensitivity is more than adequate for the plasma levels which are obtained after therapeutic doses of the drug.

Assay of Specificity. There is a negligible amount of material in normal plasma and urine which assays as dicumarol in the analytical procedure described above. The possible interference by metabolic products of the drug was examined by a distribution technic previously described by one of us.[1,2] It involves a comparison of the distribution of the substance extracted from plasma with that of the authentic substance in a two-phase system consisting of an organic solvent and water at various pH values. Dissimilar distributions indicate the presence of a substance different from the authentic compound. To escape detection a transformation product would have to have not only a similar dissociation constant but identical solubility characteristics in two solvents.

The examination in the case of dicumarol was made with heptane extracts of the pooled plasma of 2 subjects who had received the drug. The plasma was obtained 23 hours after the oral administration of a 500 mg

TABLE I.

Distribution of Dicumarol and Apparent Dicumarol Between Heptane and Water at Various pH Values.

The apparent dicumarol was obtained by extraction with heptane of the acidified plasma of 2 subjects who had received dicumarol. The compound was returned to dilute alkali. Aliquots of this solution and of an authentic dicumarol solution were adjusted to various pH values and shaken with 2 volumes of heptane. The fraction of the compound extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	Authentic dicumarol	Apparent dicumarol from plasma
1	.95	.94
6	.93	.91
6.5	.90	.87
7.0	.81	.80
7.5	.53	.52
8.0	.36	.35

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[1] Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, 1945, **158**, 705.

[2] Brodie, B. B., Udenfriend, S., and Baer, J. E., *J. Biol. Chem.*, 1947, **168**, 299.

studies, to evaluate the consequences of the observations made, in the same way as it seems difficult to understand the cause of the probable significance of light in relation to the concentration of choline in serum. It seems, however, that determinations for choline in serum might present a possibility of measuring—by a fairly simple method—the effect of light on the organism. Furthermore, the variations seem to be so large that a chemical method of determination might well be employed.

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The Pathogenicity of Bagasse, II. Effect on Rabbits of Prolonged Exposure to Bagasse.[*]

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The clinical and industrial health problems of Bagasse Disease have attracted increasing attention.[1-12] In spite of careful observation and biopsies on patients suffering of this disease, its pathogenesis remains obscure.[2]

Various factors have been suggested as etiologic agents: the particular composition of the fiber itself, fungi and micro-organisms attached to the fiber, and the high silica content. Allergic phenomena also entertained as a possible etiologic mechanism[3] seem, on the basis of experimental evidence,[4] less likely to be involved.

The problem of establishing whether the fiber itself, or the micro-organisms growing in abundance on it,[4,5] is responsible for the disease was approached experimentally by comparing the lesions produced by either untreated, autoclaved, or formalized bagasse.[4] In short term experiments a striking difference was observed. Rabbits developed a rapidly progressing, even fatal, disease after intravenous or intratracheal administration of fresh bagasse, while the autoclaved or formalized material produced only a foreign body reaction limited to the lungs. In the present report these experiments were extended in order to study the character of the lesions at longer intervals, to resolve the complex histopathology of the lesions into components that could be correlated with the various ingredients of bagasse dust, and to compare the morphology of the experimental lesions with that of the human disease.

Material and methods. For intratracheal insufflation, a procedure described earlier was employed.[4] The attempt to expose animals

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[*] Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

[†] Department of Bacteriology, Yale University Medical School; present address: Department of Microbiology, Western Reserve University Medical School, Cleveland, Ohio.

[1] Anonymous, *J. A. M. A.*, 1948, 1050.

[2] Lemone, D. V., Scott, W. G., Moore, S., and Koven, A. L., *Radiol.*, 1947, **49**, 556.

[3] Castleden, L. I. M., and Hamilton-Paterson, J. L., *Brit. M. J.*, 1942, **2**, 478.

[4] Gerstl, B., Tager, M., and Marinaro, N. A., *Arch. Path.*, 1947, **44**, 343.

[5] Hunter, D., and Perry, K. M. A., *Brit. J. Industr. Med.*, 1946, **3**, 64.

[6] Sonkin, L., Lipton, W., and Van Hoesen, D., *J. Industr. Hyg. and Tox.*, 1946, **28**, 273.

[7] Gardner, L. U., *Am. Rev. Tuberc.*, 1920, **4**, 734.

[8] Moore, M., *Arch. Path.*, 1946, **42**, 113.

[9] Browne, C. A., *J. Am. Chem. Soc.*, 1904, **26**, 1221.

[10] Hirsch, E. F., and Russel, H. B., *Arch. Path.*, 1945, **39**, 281.

[11] Sodeman, W. A., and Pullen, R. L., *Arch. Int. Med.*, 1944, **73**, 365.

[12] Koven, A. L., *Am. Rev. Tuberc.*, 1948, **58**, 55.

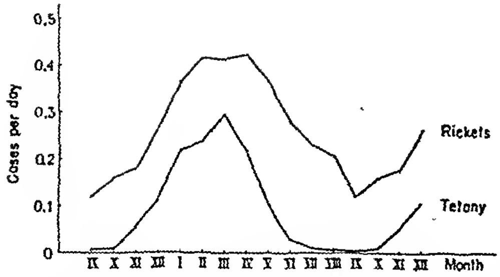

FIG. 2.

Monthly distribution of the incidence of rickets and tetany during the years 1924-35 incl. The material is from Borne-hospitalet, Fuglebakken, Copenhagen.6

biologically on intestines of guinea pigs. The accuracy of the method is 12% on single determinations, 9.4% on duplicate determinations.[4]

Fig. 1 shows the results of these determinations for choline over a 12-month period.

It will be seen that the average choline content is lowest in the month of July (0.3 mg %), the concentration being 5 times as high in the months of February and March.

The cause of these seasonal variations is not known, but we wish to call attention to the remarkable correspondence of the serum choline curves in Fig. 2, showing seasonal variations in the incidence of rachitis and tetany (Horstmann and Petersen[6]).

With the view of ascertaining the relationship between the choline seasonal curve and the curves (Fig. 2) of minimum accumulation of light,* which is secondarily manifested by deficiency of vitamin D, a few experiments were carried out in which the response of the serum choline level to irradiation was studied. Table I shows the results obtained in these experiments.

It appears from the 5 irradiation experiments that the serum choline content dropped quite considerably subsequent to about three weeks of carbon-arc light irradiation . It should be pointed out, however, that the last *3 experiments* were carried out in the latter part of April when the average choline content had already dropped.

From the present studies it seems justifiable to reckon with the possibility that the variations in serum choline content within the *various months of the year may* be related to the effect of light accumulation in the same way as is the case with vitamin D, except, *however, for showing an opposite course,* with minimum values in the winter months of February and March, instead of the summer months.

It is impossible, on the basis of the present

TABLE I.

Results Showing the Effect of Carbon Arc Light Irradiation on the Serum Choline in Five Different Persons.

	Case	Course of light treatment			Final effect
Date of initiation of irradiation and subsequent choline determinations	a	3/2/48	3/2/48	3/10/48	3/22/48
	b		3/3	3/10	4/12
	c		4/2	4/ 8	4/22
	d		4/2	4/ 8	4/22
	e		4/2	4/ 8	4/22
Minutes of exposure to carbon arc light at two day intervals	a	0	4	14	30
	b		0	4	16
	c		0	10	24
	d		0	10	20
	e		0	6	20
Serum choline (mgm %)	a	1.37	1.51	2.14	.48
	b		—	1.41	.61
	c		—	1.33	.49
	d		—	2.55	.78
	e		—	1.94	.98

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[6] Horstmann and Petersen, H., *Acta paed.*, 1947, **33**, 203.

* For full clarification of the term "light accumulation" the reader is referred to the work of Horstmann and Petersen.6 In brief, it refers to a delayed, cumulative effect of light upon the organism.

liferation and a few small round cells were seen at the periphery of some of the lesions, rendering them granuloma-like. There was little difference between the morphology of these lesions and those observed at 10-day intervals.[4]

B. Effect of ash and resins of bagasse. For the purpose of differentiating the effect of minerals present in bagasse from that of the fiber as a whole, 2 rabbits received each 10 ml of ash suspension, as described under Materials and Methods, by the intratracheal route, and were sacrificed 35 days later. Microscopic sections revealed occasional foreign body giant cells, both single or in small groups, in the interstitial pulmonary tissue. Some irregular shaped cytoplasmic defects in these cells corresponded, under polarized light, to double refractile bodies. In the pulmonary sections of one of the animals two small granulomatous lesions, composed of a few multinucleated giant cells and fibroblastic proliferation were noted; and in a section of the other animal a few alveoli plugged by young connective tissue were noted. (Fig. 1).

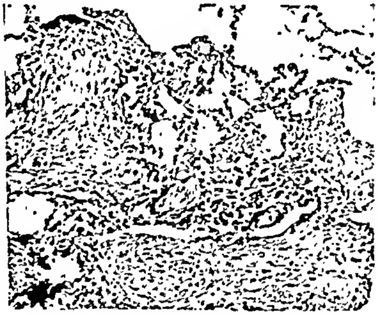

FIG. 1.
Rabbit 328. Treated intratracheally with ash of bagasse; 35th day. Lung. Masson strain. ×75.

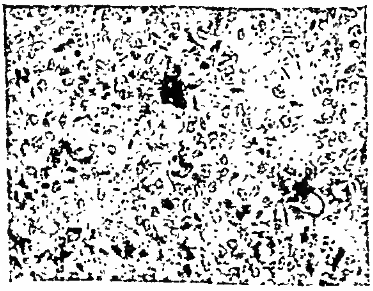

FIG. 2.
Guinea pig. Intraperitoneal injection of ash of bagasse; 90th day. Peritoneal tissue. H. & E. stain. ×165.

Intravenous administration of similar material in repeated small doses, totaling 8 ml produced occlusion of numerous capillaries by multinucleated giant cells which had formed around irregular shaped, sometimes highly refractile, foreign bodies. Similar giant cells were also found occluding lymphatics. This type of lesion was observed at 3 and 4 day intervals as well as at 20 and 35 days. In the latter group they differed only by being less numerous and by the occasional presence of a few small round cells or slight fibroblastic proliferation around the giant cells. The pulmonary changes in two rabbits, treated similarly and sacrificed at 55 days, were almost identical to those of the aforementioned ones. It is noteworthy that the other organs of these animals were free of lesions.

Three guinea pigs each received intraperitoneal injections of 2 cc of the ash suspension, and were sacrificed 90 days later. Two of them revealed granulomatous lesions attached to the visceral and parietal peritoneum, and composed of numerous large giant cells, mononuclears, and slight fibrous tissue proliferation (Fig. 2). Numerous variously shaped foreign bodies were within cytoplasmic vacuoles of these cells. None of these lesions resembled those seen in experimental silicosis.

The extent and number of lesions, as well as the intensity of the cellular reaction in the rabbits that received ash suspension were strikingly less than in the animals treated with autoclaved or formalized bagasse, although the mineral content of the material administered was a multiple of that in the former group.

Browne,[9] in an early investigation of the composition of bagasse, pointed to its high resin content. Resins are not innocuous substances, although the literature on this subject is scanty (Hirsch and Russel[10]). An attempt was, therefore, made to arrive at an appraisal of the biologic properties of the resins present in bagasse. Extracts, prepared as stated under Methods, and suspended in ethanolamine water were administered intra-

TABLE I.
Rabbits Exposed to Bagasse in Dusting Chamber.

Rabbit No.	No. of hours exposed	Duration of exposure, days	Day after last exposure that animal died (d) or was sacrificed (s)
296	270	108	11th (s)
297	270	108	21st (s)
347	30	6	1st (d)
356	72	15	1st (d)
359	90.	15	1st (d)
362	180	44	24th (s)
352	30	6	30th (s)
363	180	44	36th (s)
348	54	12	36th (s)

to a continuous and somewhat controlled flow of dust for days or weeks met with difficulty. An arrangement, similar to that recommended by Sonkin *et al.*[6] proved unsuccessful because of the hygroscopic bagasse powder rapidly becoming sticky and plugging the jet. Shaking a large amount of bagasse and maintaining the dust in the air by means of a fan or blower resulted in unequal distribution in the dusting chamber. Finally, resort was taken to a modification of the relatively simple method employed by Gardner.[7] Bagasse was finely shredded in a Waring blender and strained through a wire gauze sieve, 150 meshes per inch. Dusting was carried out for various periods during the day time. (See Table I). Approximately 15 g of bagasse were expended during 6 hours of dusting. The animals with their fur thickly coated with dust were then replaced in their cages.

To secure ash, bagasse was kept in an electric furnace at 850°F, with air blown into the oven to insure oxygenation. The resulting ash was ground in a glass homogenizer. The average yield was 5.74%. Hunter and Perry[5] as well as Koven,[12] reported 3 to 4%. The silica content of the ash was 43.34 and 43.05%.[‡] The ash of 10 g bagasse, ground with the addition of saline, was made up to 90 cc. Each cubic millimeter contained approximately 35,000 particles. Its total content of solid particles, estimated volumetrically after centrifugation, was twice that of suspension of bagasse.[4] The mineral content, however, was about 10 times that of the suspension of bagasse fiber.

An extract of the resinous substances was obtained by refluxing 5 g of shredded bagasse with 100 cc of petrol ether or benzol for 10 hours. The solvent was evaporated from the filtrate. The residue suspended well in 15 to 20 cc of water, to which 2 drops of monoethanolamine had been added.

Results. A. Intratracheal insufflation of bagasse. Rabbits studied at 20 and 35 days after the intratracheal insufflation of 10 ml of suspension of fresh bagasse showed frequent and large pneumonic lesions, with the alveolar exudate predominantly monocytic in character, though polymorphonuclears were numerous in places. The interstitium was infiltrated by small and large round cells. Fibroblastic proliferation was noted at the periphery of several lesions. Other lesions were of a foreign body granulomatous type and composed of multinucleated giant cells and mononuclears grouped together by bundles of fibroblasts. Cytoplasmic defects of irregular shape were often seen in the giant and mononuclear cells, but birefringent bodies could be identified under polarized light only in a few instances. From the pulmonary lesions of one of the rabbits (35th day) an Aspergillus was isolated on culture. In some sections radiate bodies (Moore[8]) could also be identified. Rabbits treated similarly with autoclaved or formalized bagasse and studied at identical intervals revealed lesions composed of one to several multinucleated giant and mononuclear cells, both showing cytoplasmic vacuoles. Slight fibroblastic proof lesions, may be considered together. There were many alveoli in groups of 3-20 which were filled by closely packed large round or polygonal cells, some of which could be identified as mononuclear phagocytes. Their cytoplasm contained brown dust-like material and also birefringent rods. A few multinucleated giant cells of the foreign body type and occasional polymorphonuclears were present. There was no necrosis. In pulmonary sections of rabbit 363 there was a marked thickening of the interstitium by monocytic infiltration and fibroblastic proliferation sometimes forming protrusions (Fig. 5) similar to those observed in rabbit 356.

[‡] These values were obtained on a single sample and carried out by Dr. Carl Tiedeke, New York City.

Three guinea pigs (337, 338 and 339) were also exposed in the dusting chamber for a total of 77 hours over a period of 18 days, and sacrificed 10 days later. The short interval was selected in order to observe any acute inflammatory changes which might have subsided at a later date. In the pulmonary sections some alveoli and bronchioli contained an exudate of large vacuolated mononuclear cells and an occasional polymorphonuclear. Elsewhere, large multinucleated giant cells were seen. Numerous foreign bodies were present within cells of both types. When compared with the lesions of rabbits exposed for a similar length of time, the almost complete absence of an acute inflammatory response in the guinea pig was striking.

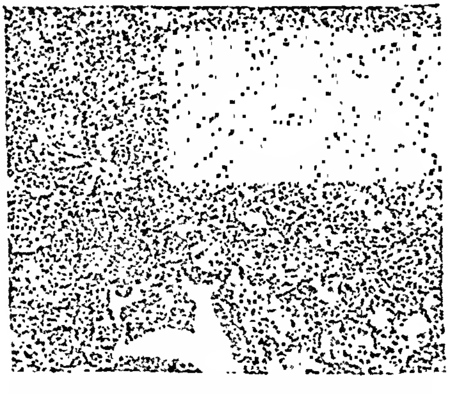

FIG. 5.
Rabbit 363. Dusted 180 hours, 36th day. Lung. H. & E. stain. ×60. Marked interstitial infiltration.

Discussion. The striking difference between lesions resulting from the administration of live bagasse, and those from bagasse dust with all living matter killed by either autoclaving or formalizing, as described first in short time experiments, was also manifest in animals kept alive for longer intervals. This is suggestive evidence that some living matter attached to the bagasse fiber, in itself an injurious agent, enhances the effect, and produces an additional pathologic change. The bagasse fiber, without living matter, elicits a long-standing foreign body reaction. Bagasse dust in the native state, when inhaled or insufflated, calls forth, in addition to the foreign body granuloma, an acute inflammatory response which, in view of its presence many days after the exposure, allows no other interpretation than that self-propagating microorganisms act upon the tissue of the host. For the rabbit, an aspergillus seems to be the pathogenic agent. It could be recovered from the pulmonary lesions 35 days after intratracheal administration of bagasse dust. This finding would support the theses of other authors[3,5,12] that fungi play an important part in the pathogenesis of the human disease.

Two components of the bagasse fiber could be demonstrated as being responsible for a long persistent tissue reaction, although the latter is of minor degree. One of them, the mineral particles, is of interest for the high silica content. But none of the lesions produced by the mineral ash resembled those of human or experimental silicosis. This would bear out the assumption of several authors that human Bagassosis is not caused by the high silica content of the bagasse fiber. The resins similarly contribute to the persistence of the lesions, as evidenced by the presence of giant cells 35 days after intratracheal insufflation. It also could be established that the resins, at least in their extracted form, do not contribute to the birefringence of the bagasse particles.

The most interesting part of the investigation was the effect of bagasse dust when inhaled by animals under conditions simulating those under which the human disease is acquired. It was soon apparent that some of the animals reacted violently to the dust with tracheally so that each of three rabbits received the total from 2.5 g of bagasse. The animals were sacrificed on the 35th day. A moderate number of multinucleated giant cells of the foreign body type were seen in the interstitial tissue of the lungs. Some of them contained large, irregularly shaped, foreign bodies which did not rotate the beam of polarized light. There was no other cellular reaction.

C. *Exposure to bagasse dust.* Animals were exposed to dust for periods indicated in Table I. For dusting rabbits 296 and 297, various mechanisms including the jet devised by Sonkin *et al.*[6] were employed. Only for the last 10 hours of exposure were these animals kept in the dusting chamber used for all other animals listed in Table I. Between the two animals there was a striking difference in type and extent of lesions. A few alveoli containing a mononuclear exudate were the only changes seen in rabbit 297. Although rabbit 296 was sacrificed 10 days later, its necropsy revealed extensive acute inflammatory changes. In the lung, numerous alveoli were filled by a polymorphonuclear and monocytic exudate with some of their nuclei showing pyknosis and karyorrhexis. Disintegrated cells were also noted in the perivascular lymphatic tissue. Foci of necrosis were present in liver and spleen.

Three animals, kept in the dusting chamber as indicated (Table I), died after 30, 72 and 90 hours of exposure. (Rabbits 347, 356 and 359). It is noteworthy that their exposure mates survived for a prolonged period. The microscopic preparations of the first rabbit revealed a hemorrhagic pneumonia; those of rabbit 359 revealed fairly numerous mononuclear cells in alveoli and in distended lymphatics. Small foreign bodies were seen in many exudate cells. Two healing myocardial infarcts may have accounted for the early death of this animal. The sections of rabbit 356 revealed an extensive pneumonic process with the exudate being polymorphonuclear in places, in others predominantly mononuclear. Many bronchioli were plugged by a similar, sometimes disintegrated, exudate. Occasional organization of the bronchiolar and bronchial exudate was found (Fig. 3). Multinucleated giant cells were infrequent. In addition to numerous small variously shaped foreign bodies there was noted an occasional club-shaped body, continuous with a fragment of a mycelial-like structure (Fig. 4). Frequently groups of large mononuclears with a foamy cytoplasm were seen both in alveoli and interstitium. Similar changes were also responsible for some polyp-like protrusions of the interstitium into the alveolar lumina. Some of the alveoli were lined by a cuboidal metaplastic epithelium.

Four animals were exposed to bagasse dust from 30 to 180 hours, and sacrificed at intervals from 24 to 36 days after the last exposure. These animals, because of the approximating interval, as well as the similarity

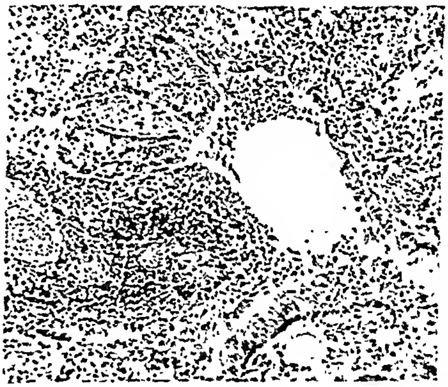

FIG. 3.

Rabbit 346. Exposed to bagasse dust for 72 hours. Died. Lung. H. & E. stain. ×100. Bronchioli plugged by exudate. Interstitial infiltration by inflammatory cells.

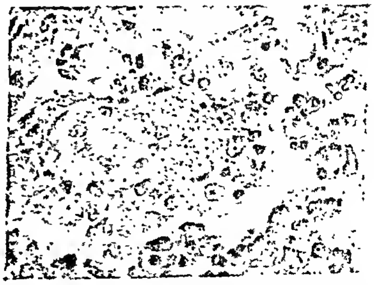

FIG. 4.

Rabbit 356. Lung. Masson stain. ×425. Spore and mycelia-like structure; foreign body giant cell.

17039

Complete Regression of Lymphosarcoma Implants Following Temporary Induction of Riboflavin Deficiency in Mice.

Herbert C. Stoerk and Gladys A. Emerson.
From the Merck Institute for Therapeutic Research, Rahway, N. J.

It has been found previously that lymphosarcoma implants show marked regression following the administration of a pyridoxine antagonist (desoxypyridoxine).[1] In recent experiments a number of other vitamin antagonists were examined for their possible effect upon lymphosarcoma (6C3H-CD) in mice of the C3H strain. It was found that the feeding of a diet low in riboflavin produced regression of established lymphosarcoma implants. This regression was enhanced by the administration of riboflavin antagonists (isoriboflavin,[2] galactoflavin.[3]).

C3H male mice 5-6 weeks of age were inoculated with lymphosarcoma (6C3H-ED)* fragments under sterile precautions. The tumor transplants were placed under the skin of the lower back. The tumors were then permitted to grow for 6 to 14 days. During this time the animals were maintained on a complete diet of natural foods. They were then divided into experimental groups and subjected to treatment as indicated in the table. At the end of the feeding periods the animals were transferred back to the stock diet. The findings summarized in the table show that in 51 control animals on a stock diet (Group I), the tumors attained an average size of 9.2 cm^3 within 20 days. After 30 days there were no survivors in this group. Mice fed for 14 days (group II) and for 26 days (group III) a diet deficient in vitamin B_2 showed marked to complete regression of the established implants within 10 days. Thirty per cent of the mice in group II and 37% in group III survived for more than 60 days, a period after which no recurrences were ever observed up to 200 days. Amounts of riboflavin required for maintenance in C3H mice, when fed to lymphosarcoma bearing animals (Group IV), were not sufficient to permit growth of the implants at the usual rate. However the rate of tumor growth did not significantly differ from that in the controls (Group I) when 8 μg (Group IX) or 10 μg (Group V) were given daily to the animals. Tumors that were permitted to grow to a larger size (Group VIII) regressed rapidly when a riboflavin antagonist was administered. The feeding of a riboflavin analogue Group VI and VII) was rendered ineffective when 100 μg of vitamin B_2 were given simultaneously. Animals surviving for more than 60 days, when reinoculated with lymphosarcoma tissue, failed to "take" the second implant. The administration of other vitamin antagonists (pyrithiamine, 3-acetyl pyridine and pteroyl aspartic acid) had no significant effect upon the lymphosarcoma implants.

Regression of lymphosarcoma transplants, produced by the administration of a pyridoxine antagonist (desoxypyridoxine),[1] occurred more rapidly than the regression observed in riboflavin deficiency. However refeeding of vitamin B_6 deficient animals with pyridoxine was followed by recurrence of the tumors. In mice in which tumor regression had been induced by transitory vitamin B_2 deficiency, the tumor did not recur when riboflavin was fed. Moreover in these mice even reinoculation was ineffective. This suggests strongly that immunization may be an important contributing factor in the permanent suppression of the lymphosarcoma transplants. In the riboflavin deficient rat antibody formation is unimpaired whereas immune responses in pyriinstant and protracted cough, and developed a rapidly progressing pneumonia. The complex morphology of the lesions found in these animals includes hemorrhagic pneumonia, bronchiolitis, with plugging of passageways by cellular exudate and debris, interstitial thickening by exudate and fibroblastic proliferation, as well as of foreign body reaction. On the other side are the animals that offered a higher resistance, possibly aided by elimination of some particles by the upper respiratory channels. These animals probably would have survived indefinitely and demonstrated the ability to resolve gradually the lesions which they undoubtedly harbored at an earlier date.

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[1] Stoerk, H. C., *J. Biol. Chem.*, 1947, **171**, 437.

[2] Emerson, G. A., and Tishler, M., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 184.

[3] Emerson, G. A., Wurtz, E., and Johnson, O. H., *J. Biol. Chem.*, 1945, **160**, 165.

* The tumor was obtained through the courtesy of Dr. W. U. Gardner, Yale University, New Haven, Conn.

Although these two groups of animals represent an interesting parallel to the selective morbidity among workers in the bagasse industry, a comparison of the morphology of the human with that of the experimental disease is hindered by the scarcity of human material available.§

Of the 2 cases that came to autopsy (Sodeman,[11] Hunter and Perry[5]) only one has been reported so far. Large spicules, as illustrated in Sodeman's case, could not be found in the tissue of animals that were exposed to the dust. Apparently particles of that size could not pass the smaller respiratory lumina of the animals. Other characteristic features are interstitial fibrosis, bronchiolitis,[5] and the presence of numerous large foamy alveolar cells filling the alveolar spaces.[11]

In the experimental lesions interstitial fibrosis was scanty. This may be due to a greater resolving power of the animal tissue or to the fact that the more susceptible animals succumbed too early (6 and 15 days) to have developed extensive fibrosis. Fibroblastic proliferation, however, was observed. Large foamy exudate cells and plugging of the bronchi were the features whereby the experimental most closely resembled the spontaneous disease.

The character of the lesions in the guinea pig differed strikingly from that in the rabbit. These represented a response to foreign bodies and lacked the acute inflammatory and progressive component, and are apparently due to the fact that the guinea pig is not susceptible to the organisms associated with bagasse.[4,5]

Conclusions. These experimental and comparative studies permit the conclusion that the inorganic part of the inhaled bagasse dust produces a long-standing tissue reaction which is primarily a foreign body response. It is unlike silicosis and amenable to healing by resolution. Superimposed on these lesions there occurs in those animals which are more susceptible to the causative microorganisms acute bronchiolitic and pneumonic changes which, if sufficiently extensive, may cause death of the animal. A similar combination of etiologic factors seems to be the most plausible explanation of the complex picture of human bagasse disease.

Summary. Bagasse irrespective of the route of administration, produces a complex and frequently progressive inflammatory reaction of the exposed animal. By employing native, in contrast to autoclaved bagasse and its extracted resins and minerals, it could be demonstrated that the fiber itself calls forth a long-standing foreign body reaction which is amenable to healing. The progressive inflammatory reaction and death of the animal, however, are due to microorganisms attached to the bagasse. In the instance of the rabbit, aspergilli are the pathogenic agents. The points of similarity of the experimental lesions to those observed in humans are discussed. None of the lesions resembled silicosis.

I wish to express sincere thanks to the Hospital Photographic Laboratory, Letterman General Hospital, San Francisco, for preparing these microphotographs.

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§ Dr. W. A. Sodeman kindly made available several slides of the pulmonary lesions of the case observed at Tulane University.

17040

Colloidal Properties of Nucleus. I. Effect of Temperature on Nuclear Viscosity in the Starfish Egg.

CLIFFORD V. HARDING. (Introduced by L. V. Heilbrunn.)

From the Marine Biological Laboratory, Woods Hole, and the Department of Zoology, University of Pennsylvania, Philadelphia, Pa.

Although the colloidal properties of the nucleus are important to an understanding of its role in cell division, very little information of a quantitative nature has been obtained from the living cell. It is evident from the observations of Gray[1] and Harris[2] that the nuclear contents of certain marine eggs are in a liquid state. Gray noted that the nucleolus in the germinal vesicle of the Echinus egg moved under the influence of gravity, and he determined the rate of its movement. Heilbrunn[3] was able to estimate the absolute viscosity by substituting the value for the rate of movement into Stokes' formula and assuming the densities of nucleoplasm and nucleolus on theoretical grounds. Harris[2] from his photographic observations on nucleolar movement calculated the absolute viscosity of the germinal vesicle of the Echinus egg to be about 10 centipoises, and he checked this value with determinations of viscosity by Pekarek's Browian movement method. He also found that a fall of the nucleolus was clearly seen in the eggs of 4 other species of echinoderms. It seems, therefore, that in the germinal vesicles of certain marine eggs, the viscosity is approximately 10 times that of water.

The purpose of this investigation is to determine the effect of temperature on nuclear viscosity in the egg of the starfish, *Asterias vulgaris*, by means of the falling nucleolus method. This method has the distinct advantage of yielding quantitative results without harming the egg in any way.

Procedure. Eggs in the immature germinal vesicle stage were used. The ovaries from freshly dissected animals were placed in sea water in fingerbowls, and the eggs, which were shed almost immediately, were kept at the temperature of running sea water (about 19°C) until used. Eggs removed from the animal for more than 3 hours were discarded. For each experiment a drop of dilute egg suspension was placed on a depression slide with a rectangular depression approximately 125 μ deep. A few strands of absorbent cotton were put in the chamber to keep the eggs in position and a coverslip lightly ringed with vaseline was placed on top. The eggs were then observed through a horizontal microscope.

After the suspension had remained a few minutes in this position, an egg was selected for observation. The microscope stage was then revolved 180° and the movement of the nucleolus through the entire diameter of the nucleus timed. In each observation the time was measured from the instant the stage was revolved until the nucleolus was observed to reach the bottom of the nucleus. This was repeated several times with each egg.

Temperature was varied by placing the microscope in a cold room to obtain temperatures between 3°C and 24°C. For higher temperatures the microscope was placed in a constant temperature box equipped with heating coils. Readings were made with the thermometer bulb directly on the stage. For each experiment the microscope was allowed to come to equilibrium with the desired temperature for 15 minutes or more.

Results. The results obtained are summarized in Table I, which shows the average number of seconds required for the nucleolus to fall at each temperature studied. When the values in the third column of Table I are plotted against temperature a straight line relation is suggested. The data were analyzed by the method of least squares. The resulting equation is:

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[1] Gray, J., *Brit. J. Exp. Biol.*, 1927, 5, 102.

[2] Harris, J. E., *J. Exp. Biol.*, 1939, 16, 258.

[3] Heilbrunn, L. V., *The Colloid Chemistry of Protoplasm*, Berlin, 1928.

doxine deficiency are suppressed.[4] It is probable that the recurrence of the tumor transplants following recovery from vitamin B_6 deficiency is due to the absence of immune bodies directed against the neoplastic tissue or its causal agent.

While pyridoxine deficiency produces marked atrophy of both normal[5] and neoplastic lymphoid tissue,[1] riboflavin deficiency affects only neoplastic lymphoid tissue. Riboflavin deficiency in the rat,[5] chick and mouse,[6] does not alter normal lymphoid tissue to a greater extent than does a comparable degree of inanition. The apparent exceedingly high requirement of neoplastic lymphoid tissue for vitamin B_2 may therefore be either a characteristic of the tumor lymphocyte or may perhaps represent a property of its causal agent, possibly a virus. The demonstration by Morris[7] that the growth of a mammary adenocarcinoma, related to a filtrable factor, is retarded by riboflavin deficiency, appears compatible with the latter possibility.

Summary. Marked regression of established lymphosarcoma (6 C3H-ED) implants occurred in all of 48 C3H mice rendered temporarily deficient in riboflavin either by the feeding of a diet low in this vitamin or by the administration of an antagonist. In most cases survival was significantly prolonged and 15 mice survived without recurrence of the tumors for more than 200 days. When animals which had survived 60 days or more were reinoculated with lymphosarcoma tissue, the second implant failed to take. Established lymphosarcoma implants in 81 control mice on a diet supplemented with adequate amounts of riboflavin, grew continuously and killed all animals within about 4 weeks.

TABLE I.

Growth of Lymphosarcoma Implants and of Survival in the Various Experimental Groups.

Group	No. of mice	No. of days on defic. diet	µg fed		Tumor size in cm³ and survival in % at											
			B_2	Anti-B_2	10 days cm³	10 days %	20 days cm³	20 days %	30 days cm³	30 days %	40 days cm³	40 days %	50 days cm³	50 days %	60 days cm³	60 days %
I	51	—	60*	0	1.4	100	9.2	86	—	0		0		0		0
II	8	14	0	0	1.5	100	<1	100	0	37	0	37	0	37	0	37
III	10	26	0	0	0.8	100	0	100	0	90	0	70	0	60	0	30
IV	10	38	5	0	1.0	100	4.9	100	7.1	70	0	40	0	20	0	20
V	10	28	10	0	0.9	100	12.6	100	14.8	70	—	0	—	0	—	0
VI	10	14	3	500‡	2.5	100	<1	100	0	60	0	50	0	40	0	40
VII	10	14	100	500	1.3	100	11.5	90	—	0	—	0	—	0	—	0
VIII	10	20	8	1000§	—†	100	14.0	90	<1	70	0	30	0	30	0	30
IX	10	20	8	0	—†	100	15.3	90	15.8	10	—	0	—	0	—	0

* Complete natural diet.
† No measurement made.
‡ Isoriboflavin.
§ Galactoflavin.

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[4] Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

[5] Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 90.

[6] Unpublished observations.

[7] Morris, H. P., *Ann. N. Y. Acad. of Sciences*, 1947, **49**, 119.

17040

Colloidal Properties of Nucleus. I. Effect of Temperature on Nuclear Viscosity in the Starfish Egg.

CLIFFORD V. HARDING. (Introduced by L. V. Heilbrunn.)
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The purpose of this investigation is to determine the effect of temperature on nuclear viscosity in the egg of the starfish, *Asterias vulgaris*, by means of the falling nucleolus method. This method has the distinct advantage of yielding quantitative results without harming the egg in any way.

Procedure. Eggs in the immature germinal vesicle stage were used. The ovaries from freshly dissected animals were placed in sea water in fingerbowls, and the eggs, which were shed almost immediately, were kept at the temperature of running sea water (about 19°C) until used. Eggs removed from the animal for more than 3 hours were discarded. For each experiment a drop of dilute egg suspension was placed on a depression slide with a rectangular depression approximately 125 μ deep. A few strands of absorbent cotton were put in the chamber to keep the eggs in position and a coverslip lightly ringed with vaseline was placed on top. The eggs were then observed through a horizontal microscope.

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[3] Heilbrunn, L. V., *The Colloid Chemistry of Protoplasm*, Berlin, 1928.

TABLE I.
Effect of Temperature on Time of Fall of Nucleolus and the Thixotropy of the Nuclear Colloid.

Temp. °C	Time for initial fall, sec.	Avg of times for subsequent falls, sec.	Ratio initial time to avg. of subsequent times	No. of experiments	Total No. observations
3.5	285	230	1.239	1	8
8.9	235	204	1.152	1	7
10.5	273	223	1.225	3	30
12.5	220	199	1.103	3	12
14	215	158	1.360	1	10
16	200	167	1.188	6	39
17	185	153	1.294	3	22
19	165	137	1.205	3	14
23	150	142	1.056	1	5
24	144	130	1.105	7	49
25	133	122	1.097	9	58
27	122	128	0.957	8	52
28	104	112	0.941	5	51
30	95	102	0.983	1	12
33-35.5	120	81*		1	10
33-37	95	95†		1	2

* Nuclear colloid gelled after tenth reading.
† Nuclear colloid gelled after second reading.

$$Y = 245.1 - 4.865\ T$$

where Y = Time for nucleolus to fall through entire diameter of nucleus (seconds).
T = Temperature (degrees C).

The line defined by this equation is shown in Fig. 1, the plotted points representing averages of all determinations for each 5 degree interval. Since viscosity varies directly with the time, corresponding values for viscosity were included on the ordinate scale. These values were calculated from Stokes' formula by substituting 7.17×10^{-4} cm for nucleolar radius, 56.6×10^{-4} cm for the distance the nucleolus falls, and 0.1 for the difference in density between nucleoplasm and nucleolus. The latter value for difference in density is theoretical. Justification for this is given by Heilbrunn[3] in his monograph.

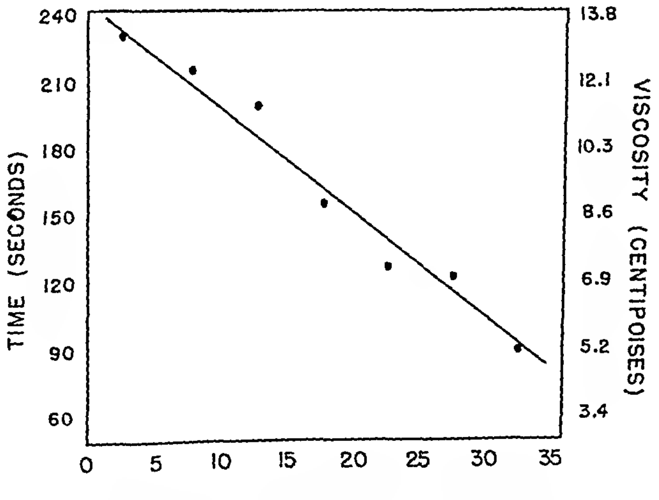

FIG. 1.
The effect of temperature on the time of fall of the nucleolus. Corresponding values for viscosity are on the right side of the graph.

TABLE II.
Results of Application of the t-test in Determining Significance of Deviations of Mean Differences from the Theoretical Value of Zero for Two Temperature Intervals.

	Temp. range (°C) 3.5-24.5	 25.0-30.0
Mean value of differences for given temperature interval	28.75	—1.4
Deviation of mean value of differences from *O*	28.75	—1.4
t value	6.268	0.489
No. of experiments	28	20
Probability of obtaining such a deviation by chance alone from the theoretical mean of *O*	0.00	0.63

Ladenburg's correction factor was used. This corrects for the effect of the nuclear membrane on the velocity of the moving nucleolus. Although the equation has not been adapted for a sphere moving within a sphere, Harris from observations on a model nucleus deduced an empirical value of 0.29 for the Echinus egg nucleus. Since the relative proportions of nucleolus and nucleus are approximately the same in the Asterias egg as they are in the Echinus egg the same value was used in these calculations. As can be seen from Fig. 1, the absolute viscosity, determined in this way, is about the same as that found in the Echinus egg by Harris. It would appear, therefore, that at least in the temperature range investigated the absolute viscosity changes by approximately 2.7 centipoises with a 10°C change in temperature. This change is evident up to temperatures of approximately 35°C. In this range the nuclear contents gel, as is evident from the third column in Table I, which indicates that the nucleolus did not move at or above this temperature.

Also, from Table I, the thixotropic nature of the nuclear colloid is suggested. In the fourth column the mean ratio of initial reading to average of subsequent readings is given for each temperature employed. If the nucleoplasm is a thixotropic colloid, this ratio should be greater than one. This is found to be the case at lower temperatures. However, the values appear to decrease with increase in temperature until a ratio close to unity is reached. This is at a temperature of approximately 25°C. Apparently, therefore, temperatures of 25°C or above abolish thixotropy.

In order to test the significance of this apparent change in thixotropy the differences between the initial readings and the averages of subsequent readings were analyzed by means of the t-test (based on Student's distribution). The mean of the differences obtained in experiments at temperatures of 25°C through 30°C proves to be insignificantly different from zero. The mean of the differences corresponding to temperatures of 3.5°C to 24.5°C, however, is significantly greater than zero. The numerical results of these calculations are given in Table II.

Discussion. There were no marked deviations from a linear relationship between viscosity and temperature within the range studied. This is unlike the cytoplasmic reaction in the Cumingia egg as found by Heilbrunn.[4] In this case the viscosity goes through a maximum value at 15°C. In some other cells, however, the cytoplasmic viscosity decreases progressively as the temperature is raised until the protoplasm gels (see Heilbrunn[3] for discussion). This is similar to the results obtained with the starfish egg nucleus. Coagulation of the starfish egg nucleoplasm occurred at approximately 35°C. For the present data, the latter value is necessarily an approximation since a complete description of heat coagulation would involve not only the temperature but also the time that the eggs were exposed. Heilbrunn[5] has shown that the protoplasm of the Arbacia egg coagulates at 31°C to 37°C, that of the Cumingia egg at higher temperatures. In both cases, the temperature coefficient of the change in rate of coagulation per degree change in temperature lies between 1 and 2. Investigations are planned to determine other properties of the nuclear colloid, particularly with regard to its radiosensitivity. The present knowledge of temperature effects will serve as a guide to the proper experimental conditions.

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[4] Heilbrunn, L. V., *Am. J. Physiol.*, 1924, **68**, 645.

[5] Heilbrunn, L. V., *Am. J. Physiol.*, 1924, **69**, 190.

Summary. By means of the falling nucleolus method it was determined that a 10°C increase in temperature lowers nuclear viscosity in the Asterias egg by approximately 2.7 centipoises. Thixotropic properties disappear at temperatures above 25°C and coagulation occurs at about 35°C.

17041

Effect of Stretch and Pressure on Stimulus Formation in the Dog's Auricle.

D. SCHERF, M. M. SCHARF, AND M. F. GOKLEN.*
From the Department of Medicine, New York Medical College.

The application of a few crystals of aconitine or of 0.05 cc of a 0.05% solution of aconitine to the auricular appendix of the exposed heart of the dog results in a regular tachycardia with a rate of 300-500 beats per minute. Invariably this tachycardia responds to faradic stimulation of the vagus nerves with an increase of rate.[1] The administration of aconitine to the site of the sinus node also leads to a tachycardia which spontaneously or following stimulation of the vagus or sympathetic nerves is transformed into auricular fibrillation.[2,3] Because of its high rate and the ease of its transformation into fibrillation the auricular tachycardia was considerd to be auricular flutter. Cooling the small area to which the aconitine has been applied stops the fibrillation (or flutter); it reappears immediately when cooling is interrupted.[2] Earlier it was pointed out that these observations are not compatible with the assumption that auricular fibrillation is due to a circus movement mechanism. An extremely rapid stimulus formation with secondary formation of reentry waves was considered the responsible mechanism.[2]

This report deals with observations made during application of stretch and pressure on the right auricle of the dog's heart in the presence of an aconitine-induced tachycardia.

Method. The technic employed was the same as in previous investigations. The heart was exposed with the dog under nembutal anesthesia and artificial respiration. Aconitine was injected into the region of the head of the sinus node. All electrocardiograms were recorded in lead II. Stretching of the auricle was accomplished by one of several methods: 1) by attaching a weighted hook to the right auricular appendix or the right auricle at the junction between the vena cava superior and the right appendix; 2) by stretching the tip of the appendix of the right auricle by means of a blunt forceps; 3) by rapid intravenous infusion of 50 cc of 0.9% saline at body temperature into the jugular vein or the vena cava superior. Pressure on the area into which aconitine had been injected was exerted by a probe. In all experiments we satisfied ourselves that the site of application of aconitine actually was the focus of stimulus formation because cooling of this site invariably resulted in a cessation of flutter or fibrillation as long as cooling continued.

Results. In 11 experiments stretching led to an increase of auricular rate and to auricular fibrillation. The fibrillation sometimes developed so quickly that the transitional period of tachycardia could not be analyzed. More often a short period of increased rate preceded the fibrillation. The duration of the

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[*] Istanbul, Turkey.

[1] Scherf, D., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 233.

[2] Scherf, D., Romano, F. J., and Terranova, R., *Am. Heart J.*, 1948, 36, 241.

[3] Scherf, D., *Am. Heart J.*, in press.

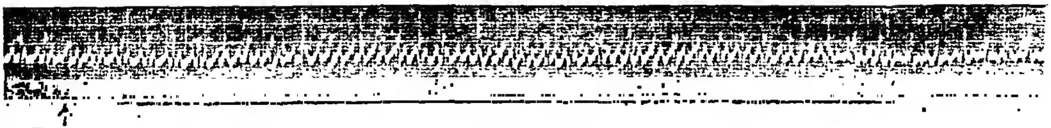

FIG. 1

FIG. 2 →

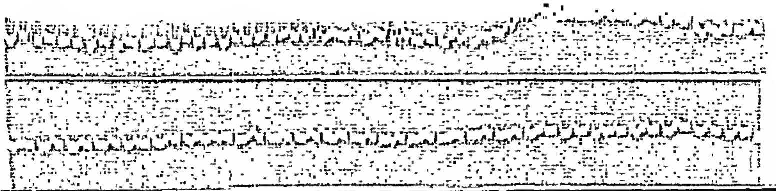

FIG. 3

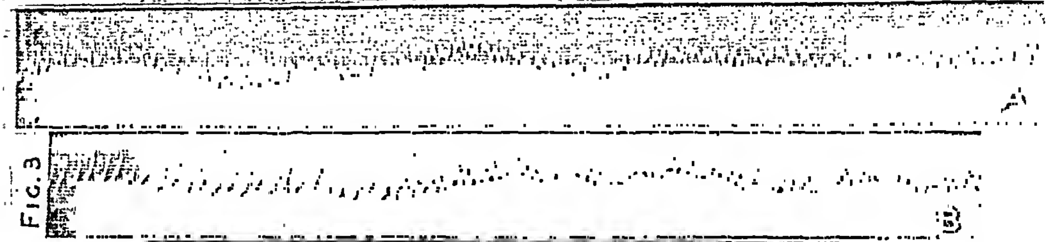

FIG. 4

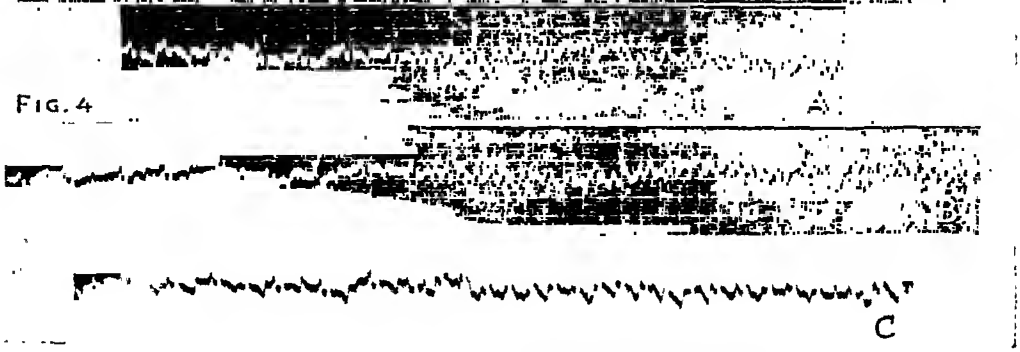

FIG. 1. Stretching of the right auricle changes auricular flutter into fibrillation.

FIG. 2a and b. The tracings are continuous. Stretching of the right auricle causes temporary auricular fibrillation and return to flutter.

FIG. 3a and b. Stretching of the right auricle causes a marked increase of the flutter rate and fibrillation.

FIG. 4a, b and c. In Fig. 4a stretching changes sinus rhythm into transient auricular fibrillation; rapid dilatation of the right auricle caused by intravenous injection has the same result (Fig. 4b). Pressure exerted on the site of injection causes auricular fibrillation (Fig. 4c).

fibrillation thus elicited varied from a few seconds to minutes.

Fig. 1 was obtained in an experiment on Sept. 23, 1947. Application of the aconitine solution to the head of the sinus node caused an auricular flutter with a rate of 460 per minute and a 2:1 A-V block. Stretching of the auricle with a weight of 20 g increased the auricular rate to 540 and finally to 600; fibrillation then suddenly appeared.

In the experiment of May 6, 1948 auricular flutter with a rate of 400 and an irregular A-V block was elicited in a similar manner. With stretching the rate increased to 500 and then to about 600; at this moment fibrillation immediately followed (Fig. 2a). Fig. 2b represents the direct continuation of Fig. 2a and shows the return of auricular flutter after approximately 10 seconds.

In the experiment of Jan. 25, 1949 the fibrillation induced by stretching lasted only about 7 seconds (Fig. 3a). The auricular rate before the stretching at the beginning of Fig. 3a measures 420 per minute. This rate was increased with stretching to about 600 and again fibrillation appeared. With the return of flutter, at the end of the tracing, the auricular rate is again 600 and gradually fell to values of around 460. The stretching was repeated about 12 minutes later with the same result. The auricular rate increased from 420 to about 660 beats per minute and auricular fibrillation appeared (Fig. 3b).

Of particular interest are the tracings of Fig. 4. In this experiment auricular stretching had converted auricular flutter into fibrillation which persisted for 58 minutes. The stretching was performed shortly after the injection of aconitine and it is possible that the stretching hastened the appearance of fibrillation which would have developed anyway. After the disappearance of fibrillation repeated stretching of the auricle by traction on the appendix *regularly elicited transient* fibrillation.

Fig. 4a shows a regular sinus rhythm with a rate of 126. Stretching caused auricular fibrillation after the auricular rate had increased from 200 to 300. Two P waves which are situated within the QRS complex just before fibrillation started, follow each other with a rate of about 600. Shortly after this fibrillation subsided, 50 cc of saline were rapidly injected into the jugular vein in order to cause acute dilatation of the right auricle. Fig. 4b obtained during this maneuver shows that after a short period of sinus node inhibition and A-V escape beats fibrillation developed. Repetition of this experiment twice yielded the same results. Finally pressure was exerted on the site of the injection of aconitine with a blunt probe and fibrillation reappeared immediately (Fig. 4c).

Similar observations were made in other experiments for a period of 15-20 minutes after the disappearance of flutter or fibrillation.

Discussion. In all 11 experiments identical results were obtained. If the auricular muscle containing the sinus node into which aconitine had been injected was stretched by traction or by rapid intravenous introduction of fluid invariably an increase of auricular rate and then auricular fibrillation were observed. Even after the aconitine induced fibrillation (or flutter) had disappeared they could be reinduced by these measures.

The appearance of rhythmic stimulus formation or the increase of rate of an already existing stimulus formation in a response to the mechanical stimulus of stretch or pressure is known to exist in sensory nerves, in motor nerves, the skeletal muscle, the heart muscle of the frog[4] and the bundles of specific fibers of the heart of the dog.[5] In lower animals, the mollusks for example, the stretch caused by cardiac filling is the physiologic stimulus for cardiac activity. Stretching and pressure facilitate depolarization of the cell membrane;[6] stronger mechanical stimuli of this kind cause local injury of the type seen during continuous chemical or electrical stimuli which also lead to rhythmic stimulus formation. On the other hand conductivity does not seem to be altered by mechanical stretching. This fact has been demonstrated for the skeletal muscle,[7] for the junctional A-V fibers of the heart of the frog[8] and finally for muscle strips from the frog's ventricle.[9] In the experiments mentioned last stretching which increased by 20% the distance between two points on which the electrodes were attached did not increase conduction time.

In view of the fact that the mechanical devices used in our experiments influence only the formation of stimuli and not the conduction of the impulses we see in these observations further justification for the assumption that in auricular flutter and fibrillation we are dealing primarily with a disturbance of stimulus formation. The rate of stimulus formation is increased by stretching and when a certain limit is reached—in our experiments a rate of about 600 per minute—fibrillation appears. There are no data available which permit us to assume that stretching causes an increased rate of conduction of a circus wave. As soon as the rate of stimulus formation reaches values of approximately 600 per minute the appearance of refractory islands of muscle makes a regular response impossible and flutter changes into fibrillation.

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[4] Gaskell, W. H., *J. Physiol.*, 1880, **3**, 48.

[5] Goldenberg, M., and Rothberger, C. J., *Arch. f. d. ges. Physiol.*, 1935, **235**, 597.

[6] Adrian, E. D., and Gelfan, S., *J. Physiol.*, 1933, **78**, 271.

[7] Schenck, F., *Arch. f. d. ges. Physiol.*, 1896, **64**, 179.

[8] Engelmann, T. W., *Arch. f. d. ges. Physiol.*, 1894, **56**, 149.

[9] Schellong, F., *Z. f. Biol.*, 1925, **82**, 451.

The appearance of fibrillation following stretching, particularly on stretching by intravenous infusion (Fig. 4b) is of interest because it may explain the attacks of paroxysmal fibrillation on sudden physical exertion. The increased filling of the right auricle particularly at the beginning of sudden severe physical exercise[10] may in a predisposed heart elicit paroxysmal fibrillation.[11-13]

We fully appreciate the fact that the assumption of a rapid stimulus formation as the cause of auricular flutter or fibrillation[2] will necessitate an answer to one pertinent objection: Why does stimulation of the vagus nerves increase the auricular rate of one type of auricular tachycardia (auricular flutter) and stop the stimulus formation during sinus rhythm or other auricular tachycardias? Auricular extrasystoles caused by the intravenous injection of minute doses of aconitine disappear during the faradic stimulation of the vagus nerves. They increase in number after the stimulation of the vagus is discontinued.[14] At the present time we are not prepared to offer an explanation for this fundamental difference. It is possible that local application of aconitine causes the establishment of a continuous stimulus; this would elicit more frequent responses with a shortening of the refractory phase during stimulation of the vagus nerve. If extrasystoles occur due to discontinuous stimulus formation vagal stimulation inhibits them.

Summary. Stretching of the auricular muscle fibers during a tachycardia induced by aconitine leads to an increase of rate and to transient auricular fibrillation. Transient auricular fibrillation (or flutter) can be elicited by stretching during a period of 15-20 minutes after the aconitine arrhythmia has subsided and sinus rhythm prevails.

Because there is much evidence that stretching changes impulse formation and not impulse conduction these results are believed to bring further evidence against the circus movement hypothesis.

The experiments offer an explanation for the appearance of paroxysmal fibrillation induced by physical exertion.

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[10] Eyster, J. A. E., The clinical aspects of venous pressure, Macmillan, New York, 1929.

[11] Hay, J., and Jones, H. W., *Brit. M. J.*, 1927, **1**, 559.

[12] Orgain, E. S., Wolff, L., and White, P. D., *Arch. Int. Med.*, 1936, **57**, 493.

[13] Jervell, O., *Acta med. Scandinav., Suppl.*, 1941, **123**, 164.

[14] Scherf, D., *Z. f. d. ges. exp. Med.*, 1929, **65**, 222.

17042

Cortical Projection of Proprioception in the Cat and Monkey.*

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In spite of the abundant evidence of the importance of proprioception for the regulation of movements in general and those elicited by stimulation of the motor cortex in particular[1-3] little is known about the action of proprioceptive impulses on the cortex.†

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* Aided by a grant from the National Foundation for Infantile Paralysis.

[1] Gellhorn, E., *Brain*, 1948, **71**, 26.

[2] Gellhorn, E., *Brain*, in press. Presented at the Minneapolis meeting of the American Physiological Society, September 1948.

[3] Hyde, J., and Gellhorn, E., *Am. J. Physiol.*, 1949.

return of flutter, at the end of the tracing, the auricular rate is again 600 and gradually fell to values of around 460. The stretching was repeated about 12 minutes later with the same result. The auricular rate increased from 420 to about 660 beats per minute and auricular fibrillation appeared (Fig. 3b).

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[4] Gaskell, W. H., *J. Physiol.*, 1880, **3**, 48.

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[6] Adrian, E. D., and Gelfan, S., *J. Physiol.*, 1933, **78**, 271.

[7] Schenck, F., *Arch. f. d. ges. Physiol.*, 1896, **64**, 179.

[8] Engelmann, T. W., *Arch. f. d. ges. Physiol.*, 1894, **56**, 149.

[9] Schellong, F., *Z. f. Biol.*, 1925, **82**, 451.

FIG. 1. Effect of passive extension of r. hindleg (knee extended to 160°, ankle to plantar flexion of 140°, duration of movement indicated by arrow) on the corticogram of the cat. 1, r, sensorimotor area; 2, l. sensorimotor area; 3, l. temporal region; 4, l. occipital region; 5, l. parietal region. Calibration in Fig. 1 to 5 300 microvolts and one second.

FIG. 2. Effect of stimulation of peripheral end of r. first sacral ventral spinal root (S_1) with condenser discharges (1.5 volts, 90 per second, upper part of figure; 1.9 volts, 90 per second, lower part of figure) on the E.C.G. of the cat. No. 1 to 5 as in Fig. 1. Duration of stimulation indicated by arrow.

FIG. 3. Effect of stimulation of r. S_1 (1.5 volts, 90 per second) on the E.C.G. of the cat before and after reinforcement by fixation of stimulated extremity in extension. I, l. motor cortex, 2, l. sensory cortex (gyrus proreus), 3, l. auditory area. Left figure without fixation; right figure, knee fixated at 150° and ankle at 130°.

FIG. 4. Effect of stimulation of r. S_1 (1.5 volts, 90 per second) on left E.C.G. of the cat. 1, sensorimotor area, 2, parietal cortex, 3, occipital cortex. R. tibialis anterior and gastrocnemius muscles tenotomized. In experiment A tendons without tension. In experiment B tendons under tension by application of 225 g weight to each tendon.

FIG. 5. Effect of stimulation of r. S_1 (1.0 volts, 90 per second) on contralateral cortex of the monkey. The numbers 8, 4, 3, 7 indicate the eye field, motor area, postcentral gyrus, and parietal lobe respectively.

That the motor cortex is involved is suggested by Bard's[5] observation that the contralateral hopping reaction is abolished in the monkey by ablation of the motor cortex. If the postcentral gyrus alone is removed this reaction is lost temporarily (Peele).[6] That the precentral gyrus plays a role in proprioception is further indicated by the fact that stimulation of posterior roots elicits, in addition to potentials in the postcentral gyrus, also changes in area 4 which are not of tactile origin (Woolsey, Chang and Bard).[7] While the present work was in progress Dawson[8] showed by means of EEG records that stretching a contralateral muscle elicited a myoclonic seizure accompanied by distinct potential changes over the central part of the skull corresponding to the contralateral area 4.

It is the purpose of this paper to report experiments which demonstrate the cortical projection of proprioceptive impulses in cat and monkey.

Method and material. The experiments were performed on 35 cats and 8 monkeys (Macaque) anesthetized with 0.45 cc dial-urethane per kilo i.p. The exposure of brain and spinal cord followed standard procedures. Cortical potentials were recorded with an Offner inkwriter after proper amplification as in previous experiments. For eliciting proprioceptive impulses several methods were used: 1. gentle passive movements of one hindleg; 2. stimulation of the peripheral end of a motor root, usually S_1 as previously described by Cooper and Creed.[9] A shielded electrode of special design was used which permitted repetition of this experiment over periods as long as 24 hours; 3. stimulation of the central end of a muscle nerve with threshold currents.

Results. I. The effect of proprioceptive impulses on the cortex of the cat, a. Effect of proprioceptive impulses induced by passive movements. Passive flexion and extension studied in 5 cats were equally effective in causing an excitation of the cortex which was

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[†] The role of proprioception in cortically induced convulsions has been studied by Gellhorn, Hyde, and Gay.[4]

[4] Gellhorn, E., Hyde, J., and Gay, J., *Arch. Internat. Pharmacodyn.*, 1949.

[5] Bard, P., *Harvey Lectures*, 1938, **33**, 143.

[6] Peele, T. L., *J. Neurophysiol.*, 1944, **7**, 269.

[7] Woolsey, C. N., Chang, H. T., and Bard, P., *Fed. Proc.*, 1947, **6**, 230.

[8] Dawson, G. D., *J. Neurol. Neurosurgery Psychiat.*, 1947, **10**, 141.

[9] Cooper, S., and Creed, R. S., *J. Physiol.*, 1927, **62**, 273, and **64**, 199.

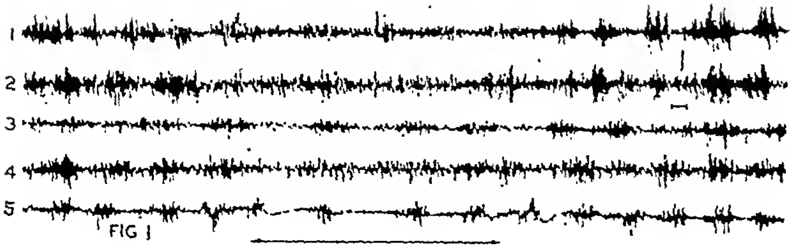

FIG 1

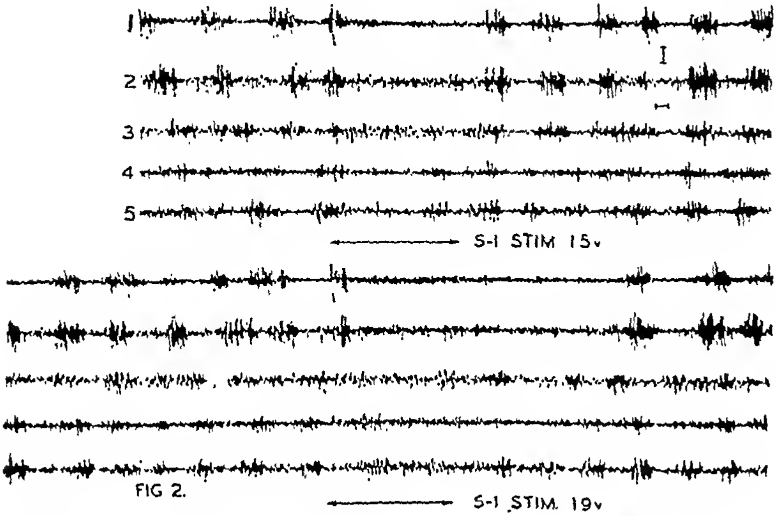

FIG 2.

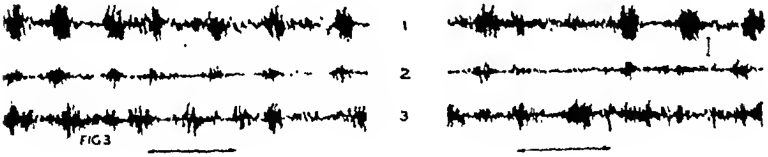

FIG 3

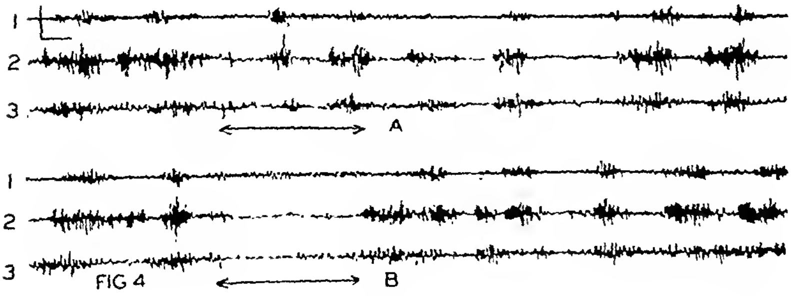

FIG 4

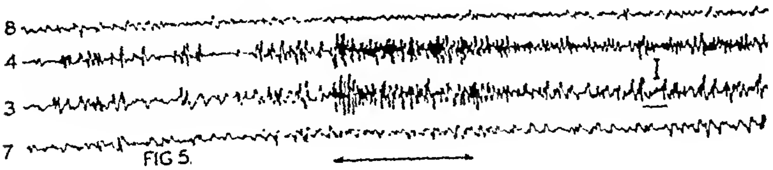

FIG 5.

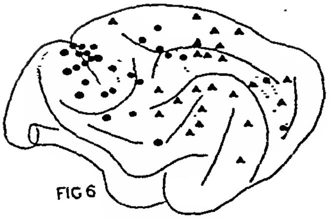

FIG 6

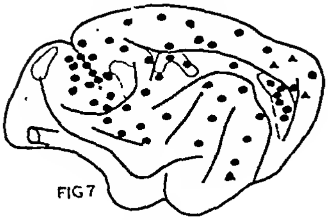

FIG 7

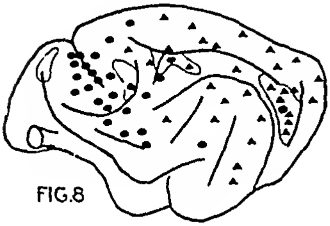

FIG.8

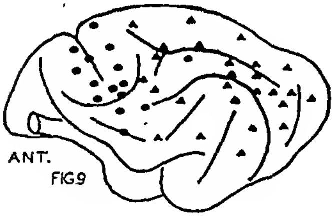

FIG 9

● EXCITATION ▲ NO CHANGE

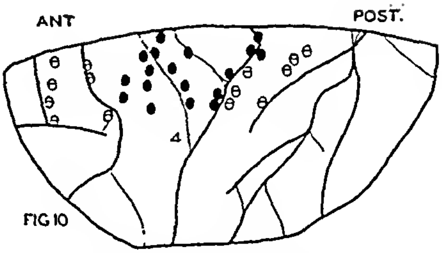

FIG 10

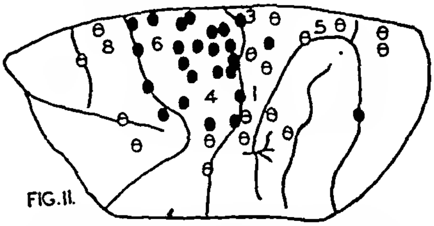

FIG. 11.

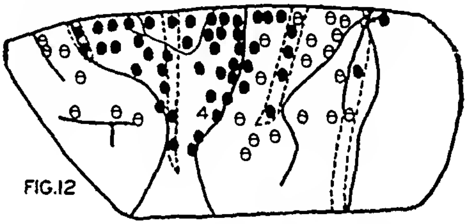

FIG. 12

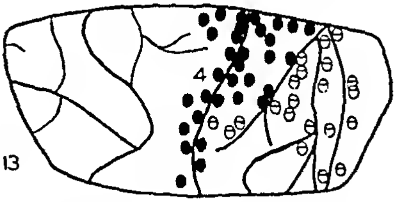

FIG 13

● EXCITATION θ NO CHANGE

FIG. 6. Projection area of proprioception in the contralateral cerebral cortex of the cat determined by ventral spinal root stimulation or passive movements of extremities in several animals. Oval symbols represent points of excitation, triangles indicate no change. Each point was verified in more than one experiment and more than one animal.

FIG. 7 and 8. Area of projection of proprioception following contralateral stimulation of S_1 in the same cat. In Fig. 7 all points showing disappearance of "Dial" potentials on stimulation are marked positive whereas in Fig. 8 only those points showing in addition increased background activity are indicated by oval symbols.

FIG. 9. Projection of proprioception in the cerebral cortex of the cat determined by stimulation of a nerve from the contralateral gastrocnemius muscle.

FIG. 10 to 13. Projection area of proprioception in the contralateral cerebral cortex of four monkeys determined by ventral spinal root stimulation (S_1).

ception tended to be a diffuse phenomenon. Thus, in the experiment shown in Fig. 7 the entire cortex was responsive except the occipital and temporal poles. However, a careful examination of the records showed that the degree of excitation was quite different in different parts of the cortex. If a distinction is made between reactions consisting of temporary disappearance of dial potentials only and those responses in which in addition the background potentials were markedly increased, it can be shown that the sensorimotor cortex is more strongly affected. In Fig. 8 a map of the same cortex is represented as characterized by the disappearance of "Dial" potentials and/or an increase in the frequency and amplitude of the background potentials. Frequently the cortical effect appeared only on the contralateral sensori-motor areas; sometimes (Fig. 1) the effect was present in both sensorimotor cortices but greater on the contralateral side. Temporal, parietal and occipital areas were consistently negative. Passive movements were repeated at intervals of more than three minutes and it was observed that the excitatory effect on the cortex gradually diminished with each successive movement. After 4 or 5 tests the changes in cortical potentials were no longer obtained.

b. *Proprioceptive impulses induced by ventral spinal root stimulation.* Stimulation of the peripheral end of S_1 which produced a strong contraction of both flexor and extensor muscles of the hindleg with condenser discharges of 1.0 to 1.5 volts (90 per second) was used in 11 cats. Electrocorticograms recorded during such stimulation showed an excitation which was greater on the side contralateral to the limb stimulated and most marked in the sensori-motor area. A stronger stimulus was required to elicit both a muscular contraction and a cortical effect than to evoke a muscular contraction alone.

Fig. 2 shows the effect of 2 different intensities of stimulation applied to S_1. The weaker stimulus produced cortical changes confined to the period of stimulation whereas the stronger stimulus greatly prolonged the excitatory effect. The "Dial" potentials disappeared in the sensori-motor areas and were unaltered in the other projection areas on stimulation of S_1 with 1.5 V, but in response to stimulation with 1.9 V some slight excitation was present outside the sensori-motor area although to a much lesser degree than in the latter. The greater amplitude of the potentials in the contralateral than in the ipsilateral sensori-motor area in both tests suggests more recruitment of neurons in the contralateral cortex.

Since it was pointed out in previous investigations that proprioceptive impulses modify cortically induced movements more effectively when the muscle contractions occur under isometric conditions the experiments with stimulation of S_1 were repeated under conditions of fixation of joints. By properly adjusting its intensity a stimulus was used which although inducing a contraction of the muscles failed to alter cortical potentials. If, however, the same stimulus was repeated while the leg was fixated in extension the "Dial" potentials disappeared in the contralateral cortex (Fig. 3). Similar results were seen with fixation in flexion.

These experiments showed that proprioceptive impulses originating in nearly isometrically contracting muscles were particularly effective in altering cortical potentials. As an illustration of primary involvement of muscle tension Fig. 4 shows that S_1 stimulation of tenotomized muscles was ineffective on the cortex while the same stimulus applied when the muscles were kept stretched with a load elicited marked potential changes in the contralateral cortex.

c. *Cortical projection area of proprioception in the cat.* The experiments reported in the preceding paragraphs have already indicated that proprioceptive impulses are projected to the cerebral cortex and involve primarily the contralateral sensori-motor cortex. Even in the recordings in which several diverse areas of the cortex were excited, the potential changes decreased with increasing distance from the sensori-motor cortex. These findings led to a more detailed study of the projection of proprioception to the cerebral cortex (11 cats) based on stimulation of S_1.

A composite map of the contralateral projection area of proprioception is shown in Fig. 6. Each point on the map was verified in 2 or more animals. The principal area excited by proprioceptive discharges was the contralateral sensori-motor cortex while temporal, parietal and occipital areas were unchanged. A few points believed to be located in areas 2s and 19s appeared to be excited.

In contradistinction to this group it was seen in several animals (degree of anesthesia ?) that the cortical response to proprioindicates a species difference or rather a different reaction due to different degrees of anesthesia is impossible to decide at present. The diffuse action noted on the cortex of the cat seems to be akin to a general awakening effect. This is interesting inasmuch as the relation of proprioceptive impulses to the state of wakefulness is well established (Kleitman).[11]

It is worthy of note that in some instances proprioceptive impulses elicited an excitation of cortical suppressor areas. Dick, Bosma and Gellhorn[12] showed recently that stretching of contractured muscles caused a temporary suppression of cortical potentials and of the responsiveness of the motor cortex to electrical stimulation.

Previous experiments have established the fact that cortically induced movements are greatly modified by proprioceptive impulses particularly if the latter are intensified by fixation of joints so that muscle contractions proceed with minimal shortening. The present work gives evidence that proprioceptive impulses alter cortical activity and that the core of this effect resides in the contralateral motor cortex. These impulses are obviously necessary for the performance of cortical proprioceptive reflexes such as the hopping reaction (Bard). However, whether the cortical projection of proprioceptive impulses is responsible for the modification of cortically induced movements is uncertain. Unpublished experiments by Loofbourrow and Gellhorn have shown that spinal nociceptive reflexes are altered in a very similar manner under conditions of proprioceptive reinforcement. Even the myotatic reflex excited by stretch in a given muscle is not restricted to this muscle but extends its effect to other muscles which appear in a similar functional grouping as seen under conditions of stimulation of the motor cortex.[13] It is therefore quite probable that proprioceptive impulses modify cortically induced activity by altering the reactivity of spinal and not that of cortical neurons and this interpretation may be valid for the previously reported effects of nociceptive impulses on cortically induced movements (Gellhorn and Thompson).[14]

It is believed that the most important cortical function of proprioceptive impulses has to do with the initiation of voluntary movements. The fact that a deafferented extremity becomes useless in higher animals although no essential change occurs in the effect of electrical stimulation of the motor cortex (Sherrington,[15,16] Hyde and Gellhorn[3]) suggests an action of proprioceptive impulses on the motor cortex. The experiments presented in this paper have provided this evidence.

Summary. The effect of proprioceptive impulses on the corticogram was studied in cat and rhesus monkey by 1. passive flexion and extension of an extremity, 2. stimulation of the peripheral end of a ventral spinal root with condenser discharges, 3. stimulation of the central end of a muscle nerve. Excitation was indicated by disappearance of "Dial" potentials and/or increase in amplitude and frequency of background potentials. That tension receptors were primarily involved was shown by the fact that a stimulus of S_1, subthreshold as far as the cortical effect was concerned, became effective if the muscles contracted isometrically or under load.

The principal area of the cortex which was excited by proprioceptive impulses was the sensori-motor area in the cat and the precentral motor cortex in the monkey. In several cats the excitation induced by proprioception was spread diffusely throughout the contralateral hemisphere except for the extreme poles, but the excitation was greatest in the

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‡ Unpublished observations.

[11] Kleitman, N., Sleep and Wakefulness, Chicago, 1939.

[12] Dick, C., Bosma, J., and Gellhorn, E., *Arch. Internat. Pharmacodyn.*, 1949.

[13] Loofbourrow, G. N., and Gellhorn, E., *Am. J. Physiol.*, 1948, **154**, 433.

[14] Gellhorn, E., and Thompson, L., *Am. J. Physiol.*, 1944, **142**, 231.

[15] Mott, F. W., and Sherrington, C. S., *Proc. Roy. Soc.*, 1895, **57**, 481.

[16] Sherrington, C. S., *Philos. Trans.*, 1893, **184** B, 641.

in Fig. 7 but only those points are marked as positive which showed increased background activity in addition to the loss of "Dial" potentials (asynchrony plus recruitment. cf. Gellhorn).[10] This figure shows a clear relation of proprioceptive impulses to the sensorimotor cortex. Again two points believed to be located in areas 2s and 19s were positive although surrounded by an unresponsive region.

Proprioceptive projection was also studied by stimulating the central end of the nerve supplying the medial head of the gastrocnemius muscle. At 1.OV a small ipsilateral reflex contraction occurred but no change in cortical potentials. Stimuli of 1.5 or 1.9V resulted in excitation of the contralateral sensori-motor cortex whereas 2.3V caused bilateral excitation of all cortical areas. For mapping purposes the weaker stimulus (1.5V) was used. The effect (Fig. 9) resembled the maps shown in Fig. 6 and 8.

II. *The effect of proprioceptive impulses on the cortex of the monkey.* a. *Proprioceptive impulses induced by ventral spinal root stimulation.* The effects of ventral spinal root stimulation on the cerebral cortex of the monkey closely resemble those seen in the cat. When a subthreshold stimulus (contraction of limb muscles without cortical change) was employed, fixation of the limb in a position of flexion or extension was usually successful in augmenting proprioceptive impulses which led to an excitation of the cortex. The most marked excitation was observed in the contralateral cortex and consisted of a great increase in frequency and amplitude of the potentials in area 4 and the closely adjacent part of the postcentral gyrus while the potentials in area 8 and 7 remain unchanged (Fig. 5). The degree of specificity of the cortical projection area is also indicated by the fact that the various parts of area 4 were unequally affected by stimulation of S_1, the change being the greatest in the medial (hindleg) area and progressively diminishing with increasing laterality.

Experiments on passive flexion or extension showed slight effects on cortical potentials restricted to the contralateral area 4.

b. *Cortical projection area of proprioception in the monkey.* The projection pattern of proprioception was much more specific in the monkey than in the cat. Excitation of the cortex induced by either passive movements, spinal root (S_1) or afferent muscle nerve stimulation was confined to the precentral motor area (area 4 and 6) and to a much lesser extent to adjacent portions of the postcentral sensory cortex (Fig. 10 and 11), In two animals (Fig. 12 and 13) the excitation extended somewhat more posteriorly in the postcentral sensory cortex to include areas 2s in one animal and area 2s and 5 in the other animal. Points anterior to area 6 and posterior to area 5 and those located in the central portion of the temporal lobe were consistently negative (no excitation). None of the monkeys showed a widespread excitation of the cortex of the type seen in the cat when the same ventral spinal root was stimulated.

The degree of excitation induced by proprioceptive impulses was greater on the contralateral motor cortex. The excitation of the ipsilateral hindleg area was very slight on stimulation of S_1 and was of the same order of magnitude as that found in the face area of the contralateral cortex.

Discussion. The uniform results obtained with passive movements, stimulation of the peripheral end of a sectioned motor root, and of the low threshold fibers of a muscle nerve known to consist of proprioceptive nerves indicate that proprioceptive nerve fibers were stimulated. Tactile receptors are not involved since in "Dial" anesthesia methods used in this paper failed to show any changes in the corticogram on tactile stimulation. Neither do nociceptive impulses play any role in these results since the effect of the above procedures is regularly eliminated by ipsilateral hemisection of the spinal cord‡ whereas the widespread cortical action of stimulation of a posterior root persists after this operation.

The effect of proprioceptive impulses on the cortex of the cat was found to be more diffuse than in the monkey. Whether this

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[10] Gellhorn, E., and Ballin, H. M., *Am. J. Physiol.*, 1946, **146**, 630.

c. Vagal reflex. In 1857 Claude Bernard showed that the bradycardia or asystole produced by stimulation of the vagus nerve could no longer be obtained after a dog had been curarized.[4] Present clinical and experimental studies have corroborated this long neglected observation.

Faradic stimulation of the vagus nerve low in the neck (10 dogs) during general anesthesia with ether or pentothal sodium caused arterial hypotension as well as bradycardia. The same stimulation after the intravenous injection of d-tubocurarine (2 units per kg) did not cause hypotension or bradycardia.

Clinically, these reflexes of circulatory depression during intrathoracic surgery due to vagal stimulation have been corrected by administration of d-tubocurarine.

Conclusion. The intravenous administration of a dose of d-tubocurarine sufficient to produce intercostal paralysis may abolish or alleviate certain autonomic reflexes that may be encountered during stimulation of autonomic pathways. Cardiocirculatory disturbances incident to stimulation of the celiac plexus, the carotid sinus, and the vagus nerve, may be so treated.

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[4] Bernard, Claude, Legons Sur les Effets des Substances Toxigues et Medicamenteures (May 30, 1856), J. B. Bailtiere et Fils, Paris, 1883, 2nd edition, p. 348.

17044

Influence of Estradiol on Alkaline Phosphatase Activity in the Genital Tract of the Rat.

ROY V. TALMAGE. (Introduced by A. A. Hellbaum.)
From the Rice Institute, Houston, Texas.

That alkaline phosphatase may play some integral part in the physiological changes occurring in the genital tract during the normal reproductive cycle and during pregnancy was first suggested by the work of Wislocki and Dempsey.[1] More recently the influence of steroids on alkaline phosphatase distribution in the genital tract of the mouse has been studied by Atkinson and Elftman,[2] and by Jenner,[3] who found that estradiol caused increased phosphatase activity in the circular musculature and in the epithelium of the glands and of the lining of the uterus and vagina of castrated animals.

The work reported here concerns the effect of estradiol on the alkaline phosphatase activity in the genital tract of the rat with special emphasis on its activity in the endometrium of the cervix.

Methods and results. Forty-two albino rats were used in these experiments. Estradiol dissolved in sesame oil, was injected subcutaneously in doses ranging from a single injection of 0.2 μg to 7 daily injections of 0.4 μg. At autopsy, the genital tract, including the vagina, cervix, and uterus, was removed and prepared after the method of Gomori[4] for histochemical determination of alkaline phosphatase. Table I summarizes experimental observations.

Notes on table. A series of 8 female rats, given 2 to 3 weeks rest after removal of ovaries, served as controls. The least amount of phosphatase activity found in any one place in the genital tract of these animals has been assigned a value of one. The amount of enzymic activity found in other locations in the same animals and in treated animals is expressed in multiple factors of this unit.

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[1] Wislocki, G. B., and Dempsey, E. W., *Am. J. Anat.*, 1945, **42**, 23.

[2] Atkinson, W. B., and Elftman, H., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 148.

[3] Jenner, R., *Nature*, 1947, **159**, 578.

[4] Gomori, G. J., *Cell. and Comp. Physiol.*, 1941, **17**, 71.

sensori-motor area. In the monkey the cortical effect was more restricted and appeared in the precentral motor cortex (area 4 and 6) and, to a lesser extent, in the adjacent part of the postcentral sensory cortex. When proprioceptive impulses were elicited in a hindleg of a monkey the area of greatest change of potentials was in a hindleg point of the contralateral motor cortex. Lesser degrees of excitation were seen in arm and face area and also in the ipsilateral leg area.

It is suggested that proprioceptive impulses to area 4 are involved in voluntary movements. Their absence accounts for the loss of voluntary movements of the deafferented limb in the monkey although no change occurs in its responsiveness to electrical stimulation of the motor cortex.

17043 P

Effect of Curare on Autonomic Reflexes.*

C. L. BURSTEIN, A. JACKSON, AND E. A. ROVENSTINE.

From the Veterans Administration Hospital, Bronx, and the Department of Anesthesia, New York University College of Medicine, New York City.

The current trend to utilize curare for muscular relaxation during surgical anesthesia has led to observations disclosing a depressor effect of curare for certain autonomic reflexes.

a. *Celiac plexus reflex.* Pressure stimulation at the celiac plexus during surgical manipulation may result in reflexogenic changes consisting of a fall in the systolic blood pressure, a marked diminution in pulse pressure, little or no change in pulse rate, and rigidity of abdominal muscles.[1,2] Administration of therapeutic doses of d-tubocurarine to 12 patients who presented such a syndrome during upper abdominal surgery, not only provided adequate muscular relaxation but also resulted in a return in the arterial blood pressure and in the pulse pressure to normal which were then sustained.

Experimental investigations in a series of 8 dogs corroborated the clinical observations. The arterial hypotension with marked decrease in pulse pressure produced by compression of the celiac ganglion were not obtained after the intravenous administration of d-tubocurarine (2 units per kg).

b. *Carotid sinus reflex.* The results observed following the intravenous injection of d-tubocurarine during carotid sinus stimulation showed similar effects.[3]

Two patients having radical neck dissection manifested characteristic carotid sinus reflex activity with severe arterial hypotension and significant bradycardia. The intravenous administration of 40 units of d-tubocurarine resulted in a restitution of the arterial blood pressure and the pulse rate within 3 minutes.

Experimental investigations in a series of 8 dogs showed similar consistent effects. Upon pulling a tape applied about the carotid bulb during pentothal sodium anesthesia there followed arterial hypotension and bradycardia. After the intravenous administration of d-tubocurarine (2 units per kg) repeated similar stimulation of the carotid sinus failed to elicit any cardiocirculatory disturbance.

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* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

[1] Burstein, C. L., and Rovenstine, E. A., *Arch. Surg.*, 1939, **35**, 599.

[2] Burstein, C. L., and Rovenstine, E. A., *Curr. Res. Anesth. and Analg.*, 1938, **17**, 134.

[3] Heymans, C., Bouckaert, J. J., and Regniers, P., Le sinus Carotidien, G. Doin et Cie, Paris, 1933.

quantitatively and are a poor criterion for estrogen activity.

Discussion. These studies confirm previous observations that injections of estrogen stimulated increased alkaline phosphatase activity in certain tissues associated with reproduction in rodents.[1,3-6] They also call attention to one of the heaviest deposits of active phosphatase in the rat—that seen in the stratified epithelium lining the lumen of the cervix. In addition, the appearance of enzyme activity after injection of estradiol is noted in the endometrial stroma of the uterus and upper cervix, in contrast to that reported for the mouse.[2] Such activity has, however, been reported for the endometrial stroma of the pregnant sow.[1]

The significance of the relationship of estradiol to alkaline phosphatase in normal reproductive physiology is not known. The enzyme is known to be concerned with calcium metabolism as well as that of phosphorus due to the interrelationship of the two metals in their action in the body. Estradiol is also associated with calcium, causing its removal from some bones while laying it down in others.[7] It is highly possible that it may also have a bearing on the calcium metabolism of certain reproductive tissues. If this be so, it is then conceivable that some of the actions of estradiol may be due to the adjustments of tissue calcium through the relation of the hormone with the enzyme.

Summary. Estradiol causes a spectacular increase in the alkaline phosphatase activity of the genital tract of the rat. This increase is most marked in the stratified epithelium lining the lumen of the cervix, but it is also apparent in the connective tissue cells of the stroma of uterus and uterine cervix and in the circular musculature of the upper part of the genital tract.

In contrast to the stratified epithelium of the cervix and of the vagina, the columnar epithelium of the uterus shows very little phosphatase activity in the presence or absence of estradiol stimulation.

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[6] Talmage, R. V., *Anat. Rec.*, 1947, **99**, 15.

[7] Gardner, W. U., and Pfeiffer, C. A., *Physiol. Rev.*, 1943, **23**, 139.

17045

Uterine Circulation Time in the Pregnant Primate, With the Uterus and Abdomen Intact.

EDWARD CLARK GILLESPIE* AND S. R. M. REYNOLDS.

From the Carnegie Institution of Washington, Department of Embryology, Baltimore, Md.

The determination of uterine circulation rate in the pregnant primate is of considerable importance. Recent investigation has led many authorities to believe that there is a relationship between abnormal uterine circulation and the late toxemic syndrome. The abnormality of circulation is considered to be uterine ischemia but this has never been demonstrated. Page[1] in discussing this theory of toxemia has stated that it is unfortunate that no one has been able to determine uterine circulation times throughout pregnancy. Moreover, Smith and Smith[2] consider that the hormonal changes they find in pre-eclampsia are the result of placental ischemia which is attributable, in turn, to uterine ischemia. The basic cause of this ischemia, which apparently develops late in pregnancy, is another matter. Page[1] lists a group of conditions such as hydromnios, multiple pregnancy, diabetes, etc., in which he feels uterine ischemia is most likely to occur and, indeed, toxemia is frequently found in such conditions

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* Fellow, U. S. Public Health Service.

[1] Page, E. W., *Obs. and Gyn. Survey*, 1948, **3**, 517.

[2] Smith, O. W., and Smith, G., *Am. J. Obs. and Gyn.*, 1949, **56**, 821.

TABLE I.
Effect of Estradiol on Phosphatase Activity.

Group	No. rats	Treatment	Uterine horns: Epithelium	Uterine horns: Stroma of endometrium	Uterine horns: Muscular layer of myometrium	Cervix: Epithelium	Cervix: Stroma	Cervix: Myometrium	Vagina: Epithelium	Vagina: Stroma
I	8	Non-injected castrated females	1	0	0	2	0	0	2	0
II	9	Castrated adult ♀, inj. with 0.4 μg estradiol daily, 4-7 days	2	1-2	1	20	2	1	10	1
III	8	Non-inj. normal adult ♀, various stages of cycle	(Only cervix studied)			Metestrous 10 Proestrous 15	2	1	—	—
IV	10 7	Castrates and Immature animals given single inj. 0.2 to 0.4 μg estradiol	"	"	"	5 (after 24 hrs)	—	—	—	—

Phosphatase activity which was localized in capillary walls is not included in this report. The cervix, in this study, is considered to begin in that part of the genital tract in which there is a sudden transition from columnar epithelium to stratified epithelium lining the lumen.[5]

Group I. Non-injected castrated females. In the endometrial epithelium of the controls, the enzyme was spread evenly throughout the cytoplasm of the cell.

Group II. Injected castrated females. In the endometrial epithelium of the uterine horns, the enzyme was localized only at the free end of the cells. In the endometrial stroma, minor amounts were found in the glandular epithelium, and further deposits were noted in the connective tissue cells. Only the circular layer of the myometrium contained any of the phosphatase.

The most marked increase in alkaline phosphatase activity was found in the cervix. On a relative basis, due to the extremely dense precipitate found in the epithelial cells, the endometrial epithelium has been given a value of 20. This precipitate was found to be distributed throughout the cytoplasm but was negligible or lacking in the nuclei.

Group III. Non-injected normal adult females. Since small amounts of estrogen probably are produced continuously by the mature rat ovary, it is not surprising that alkaline phosphatase activity was found to be high in the genital tract at all stages of the estrous cycle in the normal rat. While the distribution of enzyme was similar to that of the estradiol-injected castrate, there seemed to be a definite cyclic change in the amount of enzyme present. The least activity was noted in metestrous, the most in proestrous.

Group IV. Castrates and immature animals. As a result of a single injection of relatively small amounts of estradiol, there was a detectable increase after 24 hours in alkaline phosphatase activity in the series of both castrate and immature animals. These changes, however, were difficult to determine quantitatively and are a poor criterion for estrogen activity.

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[5] Burack, E., Wolfe, J. M., Lansing, W., and Wright, A. M., *Cancer Research*, 1941, 1, 227.

Discussion. These studies confirm previous observations that injections of estrogen stimulated increased alkaline phosphatase activity in certain tissues associated with reproduction in rodents.[1,3-6] They also call attention to one of the heaviest deposits of active phosphatase in the rat—that seen in the stratified epithelium lining the lumen of the cervix. In addition, the appearance of enzyme activity after injection of estradiol is noted in the endometrial stroma of the uterus and upper cervix, in contrast to that reported for the mouse.[2] Such activity has, however, been reported for the endometrial stroma of the pregnant sow.[1]

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Summary. Estradiol causes a spectacular increase in the alkaline phosphatase activity of the genital tract of the rat. This increase is most marked in the stratified epithelium lining the lumen of the cervix, but it is also apparent in the connective tissue cells of the stroma of uterus and uterine cervix and in the circular musculature of the upper part of the genital tract.

In contrast to the stratified epithelium of the cervix and of the vagina, the columnar epithelium of the uterus shows very little phosphatase activity in the presence or absence of estradiol stimulation.

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[6] Talmage, R. V., *Anat. Rec.*, 1947, 99, 15.

[7] Gardner, W. U., and Pfeiffer, C. A., *Physiol. Rev.*, 1943, 23, 139.

17045

Uterine Circulation Time in the Pregnant Primate, With the Uterus and Abdomen Intact.

EDWARD CLARK GILLESPIE* AND S. R. M. REYNOLDS.
From the Carnegie Institution of Washington, Department of Embryology, Baltimore, Md.

The determination of uterine circulation rate in the pregnant primate is of considerable importance. Recent investigation has led many authorities to believe that there is a relationship between abnormal uterine circulation and the late toxemic syndrome. The abnormality of circulation is considered to be uterine ischemia but this has never been demonstrated. Page[1] in discussing this theory of toxemia has stated that it is unfortunate that no one has been able to determine uterine circulation times throughout pregnancy. Moreover, Smith and Smith[2] consider that the hormonal changes they find in pre-eclampsia are the result of placental ischemia which is attributable, in turn, to uterine ischemia. The basic cause of this ischemia, which apparently develops late in pregnancy, is another matter. Page[1] lists a group of conditions such as hydromnios, multiple pregnancy, diabetes, etc., in which he feels uterine ischemia is most likely to occur and, indeed, toxemia is frequently found in such conditions as he mentions. Most often, however, no direct relationship with other pathological processes is observed. Recently a mechanism of physiological growth patterns and shapes in the uterus of pregnant rabbits has been described, aberrations from which would produce myometrial ischemia in the latter part of pregnancy.[3] Comparable changes in the growth and shape of the uterus have been observed to occur in the monkey[4] and in the human.[5] In order to test these findings in the primate and to support or negate the ischemic theory described above, we have evolved a method of studying uterine circulation with the abdomen and uterus intact, at regular intervals throughout pregnancy.

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* Fellow, U. S. Public Health Service.

[1] Page, E. W., *Obs. and Gyn. Survey*, 1948, 3, 517.

[2] Smith, O. W., and Smith, G., *Am. J. Obs. and Gyn.*, 1949, 56, 821.

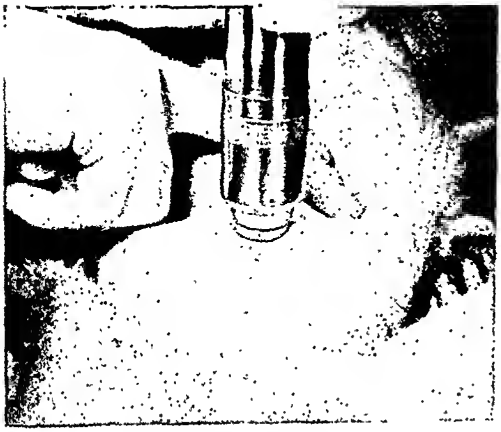

FIG. 1.
The Hypospray shown above will hold up to 1 cc of liquid. Thionine blue is being injected through the abdominal wall of a monkey 60 days pregnant.

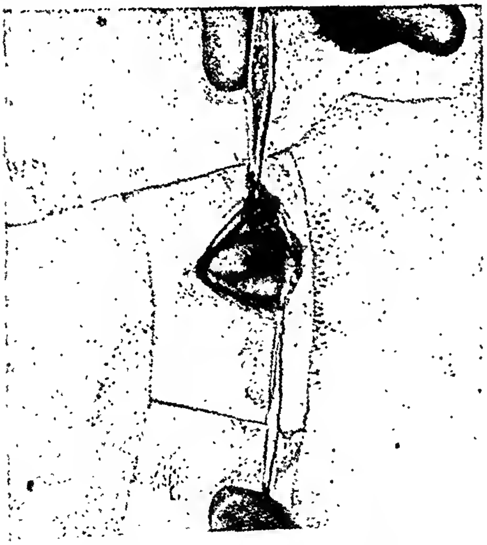

FIG. 2.
At laparotomy uterus of monkey shown in Fig. 1 is seen to contain drug injected through anterior abdominal wall. This is merely a demonstration technic.

Method. The technic described is based upon the ability of uterine muscle to clear a certain amount of dye compared to the ability of the muscle of the anterior abdominal wall to clear an equal amount of the same substance. By use of a new jet injection hypodermic called a "Hypospray" it is possible to propel drugs under the skin almost without pain. The depth of penetration depends upon the strength of the thrust developed which can be controlled by the operator. The Hypospray used in these experiments is modified from the original model described by Hingson[6] in that it is more powerful and can inject a greater amount of drug (1 cc).

The pregnant monkey uterus can be felt abdominally by the fortieth day (duration of gestation—160 days). From this time forward it is possible with a Hypospray to propel a fixed amount of Diodrast into the uterus and an equal amount into the anterior abdominal wall at another injection site. By means of serial x-rays the rate of disappearance of each injection may be observed and charted. The procedure may be performed as often as twice weekly on the same animal without evidence of damage to mother or fetus. Placental separation has not occurred even though there is nearly always an anterior implantation of a secondary placenta in monkeys. The animals gave no sign of a response to pain at the time of injection and such injections have been shown to be practically painless to humans.

A series of preliminary observations has

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[3] Reynolds, S. R. M., *Am. J. Obs. and Gyn.*, 1947, 53, 901.

[4] Gillespie, E. C., Ramsey, E. M., Reynolds, S. R. M., *Am. J. Obs. and Gyn.*, in press.

[5] Gillespie, E. C., manuscript unpublished.

[6] Hingson, R., *Analgesia and Anaesthesia*, 1947, 26, 221.

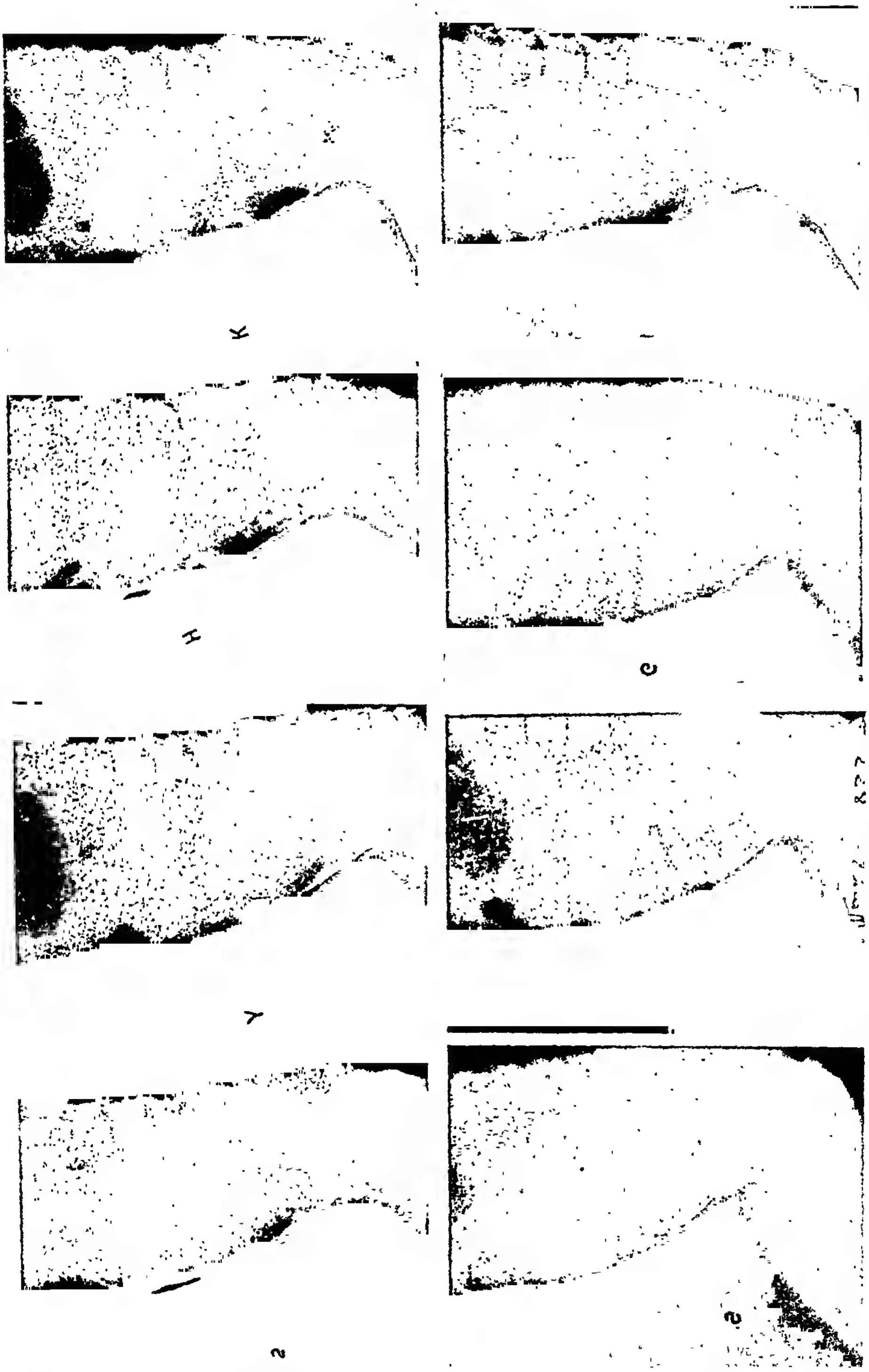

FIG. 3. The x-ray marked (2) was taken immediately following injection of 1 cc of Diodrast into the anterior abdominal wall. The dye can be seen clearly lodged in the rectus muscle just below the level of the fetal head. The x-ray marked (Y) was taken 10 minutes later immediately following the injection of 1 cc of Diodrast into the myometrium. Succeeding x-rays (H, K, S, F, E) were taken at regular intervals until both areas were cleared (I). In the series shown above clearance time for muscle was 25 minutes, and for uterus, 34 minutes. Duration of gestation was 100 days.

been made on monkeys and will be reported later. The technic has been developed in order that it may be adapted to human studies. The absence of pain, and the frequency with which it may be used make it a plan well suited to such a purpose and the investigation is anticipated. The possibility exists, therefore, that uterine clearance times may be done clinically.

Summary. One important reason for measuring uterine circulation throughout pregnancy lies in the possible relationship between ischemia of the gravid uterus and the late toxemias such as pre-eclampsia and eclampsia. We have shown a method of calculating uterine circulation by its ability to clear a radiopaque dye injected by means of a Hypospray into the myometrium. It is shown that the technic can be repeated frequently, is painless and attended by no harmful or uncomfortable sequellae. It is being adapted to human studies.

The authors are indebted to the R. P. Scherer Corp., Detroit, who manufactured the Hypospray used in these experiments, and kindly made it available to us.

17046

Inhibition by Piperidinomethyl-3-benzodioxane (933F) of Epinephrine Vasopressor Blockade Produced by Dibenzyl-β-Chlorethylamine.

JOHN C. SEED* AND ELIZABETH A. MCKAY. (Introduced by W. H. Chambers.)
From the Medical Division, Army Chemical Center, Md.

A consideration of the chemical and spatial configuration of compounds which block the pressor action of epinephrine suggests that in all probability dibenzyl-β-chlorethylamine (Dibenamine), ergotoxine, yohimbine, and the phenoxyethylamine type compounds, all block the pressor action of epinephrine by acting on the same mechanism. 933F and ergotoxine have been shown to compete with epinephrine over a wide range of concentrations in isolated physiological systems.[1,2] According to theoretical considerations, ergotoxine, yohimbine, and the phenoxyethylamine type compounds all compete with epinephrine for a receptor of the amine-alcohol configuration in epinephrine. The blocking of this receptor is believed to block the pressor action of epinephrine. Dibenamine is thought to combine chemically with, or near, this same amine-alcohol receptor.

It has been shown by Nickerson and Goodman[3] that the blocking action of Dibenamine is irreversible, in that large doses of epinephrine cannot break through its blocking action and cause a pressor response. This irreversible blocking action persists for 3-5 days after the administration of Dibenamine, by which time more epinephrine receptor is presumed to have been formed. These same authors point out that during the first hour or so after its administration Dibenamine decomposes to a physiologically inactive compound and that all the effects of Dibenamine are due to that fraction which combined with the epinephrine receptors before decomposition occurred. If during this first hour the epinephrine receptors are occupied by a compound which blocks reversibly the action of epinephrine, the Dibenamine should be unable to combine with the receptors and on removal of the reversibly-blocking compound epinephrine should give a normal pressor response. 933F is just such a reversibly-blocking compound which in doses of 5 mg/kg blocks the vasopressor action of epinephrine for 4-5 hours.

Methods. Dogs of either sex under light sodium pentobarbital anesthesia were used for all experiments. The carotid blood pressure was recorded by a mercury manometer. Injections were made into an exposed femoral vein. The 933F was dissolved in a few cc of saline. The Dibenamine was made up 5-20 minutes before use in about 20 cc of saline made strongly acid with hydrochloric

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* Captain, Medical Corps.

[1] Abdon, N. O., Hammarskjold, S. O., *Acta Physiol. Scand.*, 1940-41, **1**, 85.

[2] Gaddum, J. H., *J. Physiol.*, 1926, **61**, 142.

[3] Nickerson, M., Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

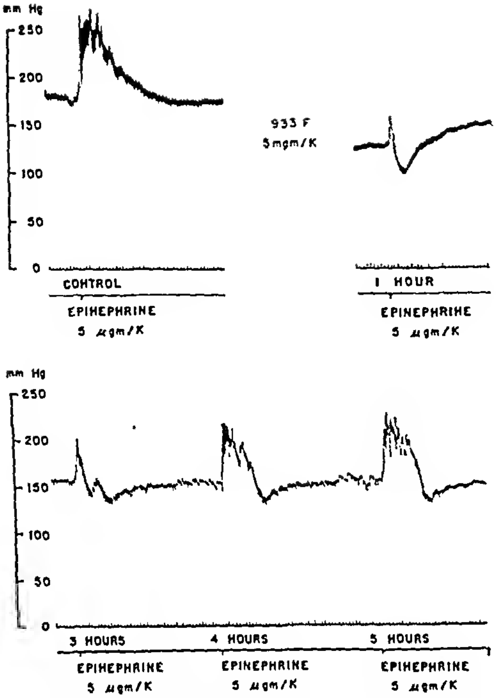

FIG. 1.

Dog, 10.2 kg, female. Sodium pentobarbital anesthesia 30 mg/kg. From top to bottom: carotid blood pressure, 6-second time intervals, injection marker. The 5 blood pressure responses to 5 μg/kg of epinephrine are (reading from left to right): one of 3 control responses obtained before the injection of 933F, and respectively, the responses obtained 1, 3, 4, and 5 hours after the injection of 5 mg/kg of 933F.

acid. The epinephrine used was that available commercially in 1 cc ampules and was diluted 1:10 with saline before injection. All animals were first tested for their response to 3 successive doses of 5 μg per kg of epinephrine. The 933F or Dibenamine was then given and the response of the carotid pressure to 5 μg/kg of epinephrine recorded at hourly intervals thereafter. When both 933F and Dibenamine were injected, the administration of the former drug preceded that of the latter by 3 minutes.

Results. Fig. 1 is a record of the tracings obtained with 5 mg/kg of 933F. By the end of 5 hours the magnitude and duration of the pressor response to 5 μg/kg of epinephrine has returned almost completely to that of the responses obtained before the injection of 933F. Fig. 2 is a record of the tracings obtained with 10 mg/kg of Dibenamine. By the end of 5 hours epinephrine still causes a marked depressor response and there are no signs of return of the initial pressor response. Fig. 3 is a record of the tracings obtained when 5 mg/kg of 933F was given followed in 3 minutes by 10 mg/kg of Dibenamine. The recovery of the pressor response to epinephrine follows essentially the same course as that found after the injection of 933F alone. There is no evidence that Dibenamine exerted any action whatsoever.

In other experiments 5 mg/kg of 933F was given and followed by an infusion of 0.2 mg/kg/min. of 933F for one hour. Recovery of the pressor response to 5 μg/kg of epinephrine was far from complete at the end of 7 hours; however, good pressor responses were obtained from 50 μg/kg of epinephrine. The administration of 20 mg/kg of Dibenamine 3 minutes after the initial dose of 933F de-

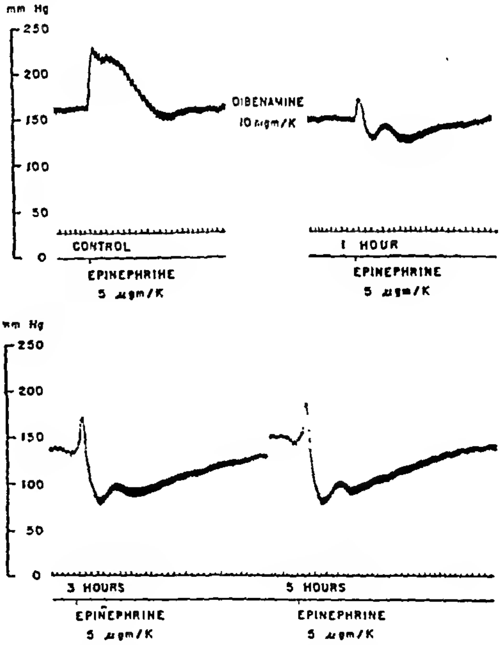

FIG. 2.

Dog, 11.8 kg, male. Sodium pentobarbital anesthesia 30 mg/kg. From top to bottom: carotid blood pressure, 6-second time intervals, injection marker. The 4 blood pressure responses to 5 μg/kg of epinephrine are (reading from left to right): one of 3 control responses obtained before the injection of Dibenamine, and respectively, the responses obtained 1, 3, and 5 hours after the injection of 10 mg/kg of Dibenamine.

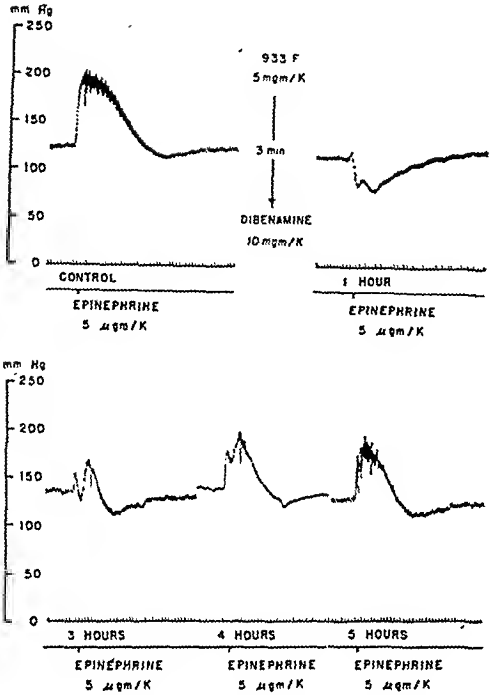

FIG. 3.

Dog, 13.8 kg, female. Sodium pentobarbital anesthesia 30 mg/kg. From top to bottom: carotid blood pressure, 6-second time intervals, injection marker. The 5 blood pressure responses to 5 µg/kg of epinephrino are (reading from left to right): one of 3 control responses obtained before the injection of 933F and Dibenamine, and, respectively, the responses obtained 1, 3, 4, and 5 hours after the injection of 5 mg/kg of 933F followed in 3 minutes by 10 mg/kg of Dibenamine.

pressed only slightly the rate of recovery of the pressor response to epinephrine from that obtained with 933F alone.

Discussion. The fact that 933F prevents Dibenamine from blocking the vasopressor effect of epinephrine may be interpreted in either of two ways. 1. The 933F may aid in the destruction of Dibenamine by either chemically combining with the Dibenamine or enabling something else to do so. 2. The 933F may prevent the Dibenamine from combining with the epinephrine receptors by protecting the receptors. From theoretical considerations and experiments now in progress the latter is considered to be the more likely explanation.

Summary. 5 mg/kg of 933F will prevent 10 mg/kg of Dibenamine from blocking the vasopressor effect of epinephrine in the dog.

The 993F used in these experiments was supplied by Dr. John E. Howard, who obtained it through the courtesy of E. Fourneau.

17047

Contamination of Commercial p-Aminohippuric Acid with p-Aminobenzoic Acid.

GEORGE E. SCHREINER, LAURENCE G. WESSON, JR., AND W. PARKER ANSLOW, JR.
(Introduced by Homer W. Smith.)

From the Department of Physiology, New York University College of Medicine, New York City.

While investigating the oral administration of p-aminohippuric acid (PAH) in man for the purpose of renal clearance measurement, it was noted that different samples of PAH, when administered according to the same routine, gave markedly different concentrations of chromogen in the plasma. PAH was determined by the method of Smith *et al.*[1] in a cadmium sulfate filtrate of plasma.

The plasma concentration of chromogen following the ingestion of 5-6 g of material in divided doses averaged 2.4 mg % (expressed as PAH) in 23 subjects receiving lot No. 12322* while the plasma concentration of chromogen averaged 0.22 mg % in 14 subjects receiving lot No. 236518.* Four tests were then performed on 2 subjects so that each received each lot of PAH under identical conditions. Lot No. 12322 gave an average plasma concentration of chromogen of 2.76 mg % in one subject and 3.05 mg % in the second, whereas lot No. 236518 gave an average plasma concentration of 0.112 mg % in the first subject and 0.329 in the second. A third subject, started on lot No. 12322 by mouth attained a plasma concentration of 2.4-3.6 mg %. When he was changed to lot No. 236518, the plasma concentration fell to 0.41 mg %. A 50 to 50 mixture of the 2 lots produced a plasma concentration about midway between the figures produced by the compounds when administered separately.

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[1] Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

* National Aniline Division, Allied Chemical and Dye Corporation.

To rule out the possibility that one compound was being metabolized, a number of experiments were performed on dogs, contrasting parenteral with oral administration. The two lots gave essentially the same plasma concentration when administered parenterally. However, a marked difference between them appeared after oral administration.

In identical experiments, lots No. 12322, No. 236518 and p-aminobenzoic acid (PAB) were compared on oral administration. Again the two lots of PAH produced a marked difference in plasma chromogen. In the dog receiving an equivalent dose of PAB, plasma chromogen concentration successively increased from 3.18 mg % at 10 minutes to 14.6 mg % at 51 minutes, with a slow decline apparent after the latter time. The plasma chromogen values in this dog were as much as 100 times as great as those produced typically by lot No. 236518.

Chemical studies. Intensity of color development. Lot No. 12322 yielded about 5% greater color per unit weight at a wave length of 540 mμ. Absorption curves from 400 to 600 mμ on a 0.1 mg % solution of each lot were identical except for the difference in intensity noted above.

Titration curve. 10 cc aliquots of 100 mg % solution prepared with carbon dioxide-free water were titrated with 0.0107 N barium hydroxide solution and the hydrogen ion concentration determined with the Cambridge pH meter. Lot No. 12322 had about 5% more available acid than lot No. 236518.

Solubility curves. Increasing quantities of each lot were placed in tubes with a measured amount of water and shaken in a constant temperature water bath at 24.6°C until equilibrium was attained. The concentration of acid in the liquid was then determined by titration with barium hydroxide. Lot No. 12322 showed increasing solubility with increasing quantities of excess substance, indicating the presence of a mixture, whereas lot No. 236518 showed a sharply breaking solubility curve with constant solubility in the presence of excess substance, indicating a relatively pure compound.

By methods of approximation it was estimated that impurities present in lot No. 12322 comprised between 17 and 31% by weight.

Infra-red absorption spectra. Infra-red absorption spectra on the above two lots and on a third lot (No. 12738) confidently known to be pure PAH,[†] showed identical absorption bands with correct characteristics of a p-aminophenyl substitution. M-aminohippuric acid showed a quite different and characteristic absorption spectrum.

The melting point (uncorrected) of lot No. 12322 was 165-169°C, of lot No. 236518, 200-202°C; lot No. 12738, 196-198°C; a 50-50 mixture of lots No. 236518 and 12322 melted at 182-185°C; a mixture of lots No. 236518 and 12738 melted at 186-188°C, and a mixture of lots No. 12322 and 12738 at 159-164°C. The recorded melting point for PAH is 198.5°C,[2] and for PAB, 187°C.[3]

Extraction data. 22 g of lot No. 12322 were extracted with ether and yielded 15 g of PAH, 4 g of PAB, and 1 g of unresolved residue. The PAB was identified as such by its melting point (183-185°C) and the fact that the melting point was not depressed by mixture with authentic PAB. The nitrogen content was found to be 10.2-10.4°C (theoretical for PAB is 10.4). The acid equivalent was 135 (theoretical 137). The preparation had the typical crystalline form of PAB.

[†] Kindly supplied to us by the National Aniline Division, Allied Chemical and Dye Corporation.

[2] Cohen, P. P., and McGilvery, R. W., *J. Biol. Chem.*, 1946, **166**, 261.

[3] Beilstein, *Handbuch der Organischen Chemie*, 1931, **14**, 419.

The PAH extracted above melted at 198-200°C, contained 14.1-14.4% nitrogen (theoretical is 14.4), and had an acid equivalent weight of 189-195 (theoretical is 194.2).

Purification of an aqueous iso-electric solution can be effected by extracting with ether and evaporating the ether to dryness. The residue *dissolved in hot water* yields crystalline PAB on cooling. The ether-extracted aqueous solution can be crystallized directly to give PAH which may be recrystallized from absolute alcohol.

Discussion. We conclude from the above data that lot No. 236518 is essentially pure PAH, whereas lot No. 12322 contains approximately 23% of PAB. Incidentally, it is demonstrated that PAB is rapidly absorbed from the intestinal tract from dog and man, whereas PAH is slowly and poorly absorbed.

PAH is made commercially by the conjugation of p-nitrobenzoyl chloride with glycine, the nitro group being subsequently reduced. Failure to effect complete conjugation, or hydrolysis during reduction, will yield PAB. Since the clearance of PAB is less than the creatinine clearance in dogs,[1] the presence of a significant quantity of this substance in PAH would introduce a large error into the determinations of the renal plasma flow. The Medical Division of Sharp and Dohme, who prepared ampouled PAH for clinical investigation, report that lot No. 12322, which we find to be contaminated with PAB, has never been marketed, and they are now examining all samples for significant contamination by PAB.

Summary. P-aminobenzoic acid is rapidly absorbed from the gastrointestinal tract in dog and man while p-aminohippuric acid is poorly absorbed.

One commercial lot (National Aniline No. 12322) of p-aminohippuric acid was found to be contaminated with 23% of p-aminobenzoic acid.

None of the contaminated material, to our knowledge, has been marketed in ampouled form for clinical investigation and an adequate control is now being maintained on the purity of clinical material.

We are indebted to Joanne Baker Schreiner for technical assistance and to Dr. Conrad Dobriner of the Sloan-Kettering Institute, New York City, for determining the infra-red absorption spectrum of the several compounds studied.

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17048 P

Recovery of Isotopic Succinate from Urine of Rats Administered Isotopic Acetate.

JUI SHUAN LEE AND NATHAN LIFSON.

From the Department of Physiology, University of Minnesota Medical School, Minneapolis.

To demonstrate that a given pathway is involved in the metabolism of an administered isotopic compound, it would appear essential to determine the isotopic composition of the assumed intermediates in the pathway. Evidence is accumulating that the Krebs or tricarboxylic acid cycle, developed mainly on the basis of *in vitro* evidence, actually operates in the intact animal, and that this scheme participates in the metabolism of acetate.[1-4] In the present investigation it was found that after the administration of isotopic acetate to rats, labeled carbon is found in succinate recovered from the urine, and that the location of isotope in this intermediate of the tricarboxylic acid cycle corresponds to the distribution predicted from the reactions of the cycle.

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[1] Krebs, H. A., and Johnson, W. A., *Biochem. J.*, 1938, **32**, 113.

[2] Lorber, V., Lifson, N., and Wood, H. G., *J. Biol. Chem.*, 1945, **161**, 411.

[3] Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., *J. Biol. Chem.*, 1948, **176**, 1263.

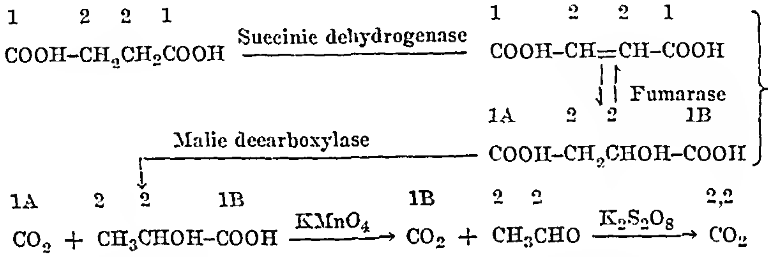
[4] Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, 1945, **157**, 749.

Methods: Two ml of 0.5 M sodium malonate per 100 g of body weight was administered subcutaneously and 1.10 mM of $CH_3C^{13}OONa$ (6.10 atom % excess C^{13} in the carboxyl group) per 100 g of body weight was fed by stomach tube to fasted rats weighing 120 - 200 g. The malonate was injected to augment urinary succinate excretion.[1] The acetate dose was repeated at the end of approximately 8 and 16 hours. The urine was collected in 20% H_2SO_4 for 24 hours, except during periods when respiratory carbon dioxide was being sampled.

The urine was extracted with ether in the presence of sodium bisulfite in order to bind keto acids. The ether extract was distilled with steam and the residue oxidized with acid permanganate. Succinic acid was extracted with ether from the oxidation mixture and purified by repeated silver precipitations.[5] The melting point of the white crystalline product was 180° - 185° and that of the Eastman Kodak product 185° - 187°.

The succinic acid recovered from the urine was converted into a mixture of fumaric acid and malic acid with succinic dehydrogenase prepared from pigeon breast muscle.[6] Fumaric and malic acid were extracted with ether and subjected to the action of an acetone powder of *Lactobacillus arabinosus* prepared by Ochoa's procedures[7] with the exception that fumaric acid instead of malic acid was used in the culture medium. The former proved to be more effective in developing fumarase as well as malic decarboxylase activity. This enzyme preparation converts quantitatively the mixture of fumarate and malate into lactic acid and CO_2.

The lactic acid thus formed was oxidized by permanganate into aldehyde and CO_2. The aldehyde was oxidized by potassium persulfate to CO_2.[8] The overall degradation scheme may be summarized as follows:



At each step, the yields of degradation products from urinary succinate compared satisfactorily with theoretical expectations and with those from authentic succinate.

Results and discussion. From the data in Table I, it will be noted that after the administration of $CH_3C^{13}OOH$, $C^{13}OOHCH_2CH_2C^{13}OOH$ is recovered from the urine. This is the result predicted from metabolism of acetate via the tricarboxylic acid cycle, by condensation with oxalacetate.[9] Since appreciable amounts of C^{13} also appeared in the respiratory CO_2 (.02 - .55 atom% excess, depending on the time interval between sampling and acetate feeding), and since CO_2 fixation would also be anticipated to yield carboxyl labeled succinate,[9] it is not possible on the basis of these results alone to evaluate the extent to which the latter mechanism accounts for the observations. The small but significant difference in C^{13} concentration between the 2 carboxyl carbons (fractions 1A and 1B), which theoretically should be equal because had the typical crystalline form of PAB.

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[5] Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, 1942, **142**, 31.

[6] Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques, 1945, p. 144, Burgess Publishing Co.

[7] Ochoa, S., personal communications; Korkes, S., and Ochoa, S., *J. Biol. Chem.*, 1948, **176**, 463.

[8] Osborn, O. L., and Werkman, C. H., *Ind. and Eng. Chem. Anal. Ed.*, 1932, **4**, 421.

[9] Wood, H. G., *Physiol. Rev.*, 1946, **26**, 198.

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17048 P

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From the Department of Physiology, University of Minnesota Medical School, Minneapolis.

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[1] Krebs, H. A., and Johnson, W. A., *Biochem. J.*, 1938, **32**, 113.

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[4] Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, 1945, **157**, 749.

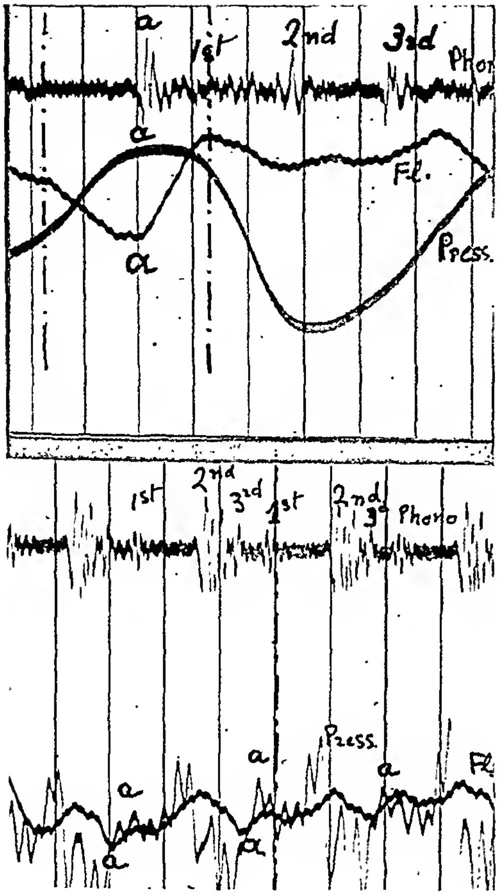

FIG. 1.
Tracings of the right auricle of two dogs. From above: phonocardiogram, fluorocardiogram, pressure tracing (electromanometer).
(A) Rubber catheter.
(B) Woven catheter.

phone applied to the left chest. Pressure tracings were obtained by introducing either a 14 French rubber catheter or a conventional, woven catheter into the right cardiac chambers through the right external jugular vein under fluoroscopic control.

Results. The heart sounds, the fluorocardiogram of the border of the right auricle, and a pressure tracing from the right auricle are recorded in the first figure.

The fluorocardiogram presents a deep negative wave in presystole (contraction of the auricle), a sharp rise at the beginning of systole, and a slower drop during systole. The pressure tracing with the rubber catheter presents a slow rise in early diastole; a more rapid rise in presystole (contraction of the auricle) and a sharp drop in systole. The *presystolic drop* in the fluorocardiogram is simultaneous with the *presystolic rise* of the pressure tracing. The systolic changes, due to the effects of ventricular systole, are revealed by a simultaneous drop in both tracings. The tracing recorded by means of the woven catheter presents multiple vibrations, 2 of which coincide with the presystolic wave of the fluorocardiogram (Fig. 1B).

In the second figure, the heart sounds, a

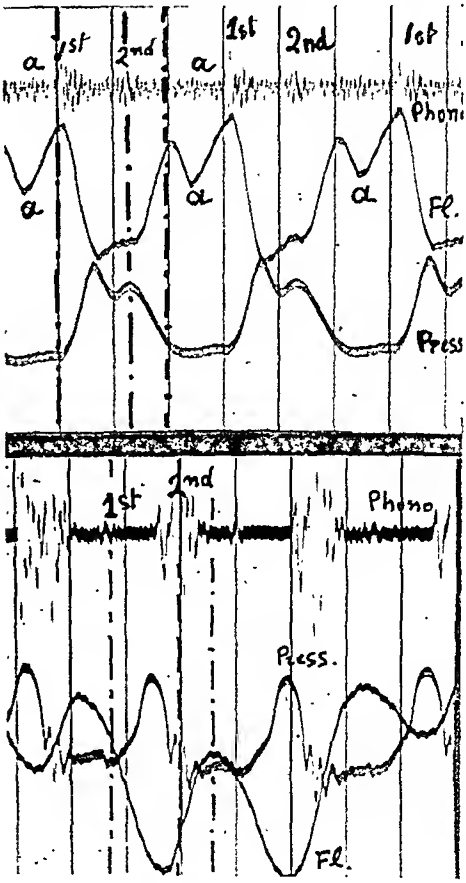

FIG. 2.
Tracings of the right ventricle of 2 dogs. From above: phonocardiogram, fluorocardiogram, pressure tracing.
(A) Rubber catheter.
(B) Woven catheter.

TABLE I.
Distribution of C^{13} in Urinary Succinic Acid After Administration of Carboxyl Labeled Acetate to Rats. C^{13} Values Are in Atom % Excess.

Exp. No.	Rat No.	Labeled acetate fed mM/100 g	C^{13} in whole succinic acid molecule	C^{13} in degradation fractions: Carboxyl carbons 1A	Carboxyl carbons 1B	Methylene carbons 2,2
I	1	1.1	0.15			
II	2	3.4	0.14			
III	3-8	3.4	0.10	0.21	0.17	0,00

succinate and fumarate are both symmetrical molecules, is interpreted as due to the oxidation of a certain amount of extraneous carbon by permanganate, but other explanations are possible.[10]

These experiments are being extended.

Summary. After the administration of malonate plus carboxyl-labeled acetate to rats, there has been recovered from the urine carboxyl-labeled succinate, an intermediate of the tricarboxylic acid cycle. This result is consistent with and additional evidence for the metabolism of acetate via the tricarboxylic acid cycle in the intact mammal.

We wish to thank Mr. Richard E. Halsted and Mrs. Ruth Boe for performing the isotopic analyses under the supervision of Dr. A. O. C. Nier, Mrs. Shirley L. Michel for valuable technical assistance, and Dr. S. Ochoa for kindly supplying us with cultures of *L. arabinosus.*

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[10] Calvin, M., and others. Isotopic Carbon, 1949, p. 192, John Wiley & Sons, Inc.

17049

Simultaneous Fluorocardiography and Recording of Intracardiac Pressure.

ALDO A. LUISADA AND FELIX G. FLEISCHNER.
From the Department of Radiology, Beth Israel Hospital, Boston.

Various studies of clinical fluorocardiography have been published in the last three years.[1-5] This method is based on the photoelectric recording of the motion of selected points of the cardiovascular contour as revealed by the fluoroscope.

Although simultaneous recordings of other graphic tracings (electrocardiogram, carotid tracing, phonocardiogram, etc.) have supported the reliability of the fluorocardiogram, an incontrovertible demonstration of the accuracy of the latter has been lacking. For this reason, fluorocardiograms of the right auricle or the right ventricle, simultaneous with tracings of pressure in the corresponding chamber, have been taken in the anesthetized dog.

Materials and methods. A Sanborn electrokymograph and a Sanborn electromanometer were used. The former is a standard apparatus for fluorocardiography; the latter is a new apparatus, based on the strain gauge principle, which permits the recording of variations of pressure with controlled amplification. A Sanborn tri-beam cardiette was employed for the transcription of the tracings so that both tracings were recorded by two separate channels while a third recorded the heart sounds through a stethoscopic micro-

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[1] Henny, G. C., and Boone, B. R., *Am. J. Roentgen.*, 1945, **54**, 217.

[2] Luisada, A. A., Fleischner, F. G., and Rappaport, M. E., *Am. Heart J.*, 1948, **35**, 336 and 348.

[3] Luisada, A. A., and Fleischner, F. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 436; 1948, **67**, 535; 1948, **69**, 23.

[4] Luisada, G. C., and Fleischner, F. G., *Am. J. Med.*, 1948, **4**, 791.

[5] Luisada, G. C., and Fleischner, F. G., *Acta Card.*, 1948, **3**, 308.

TABLE I.
Influence of Desoxycorticosterone on Excretion of Potassium in Adrenalectomized Male Rats.
(Experiments run 24 hours after adrenalectomy.)

	Control		Experimental				
KCl admin. μg/g B.W.	Mean B.W., g ± S.E., (No.)	Mean K excretion, % ± S.E.	Desoxy-corticosterone admin., μg	Mean B.W. g ± S.E. (No.)	Mean K excretion, % ± S.E.	Change, %	t
20	126 ± 4 (11)	6.78 ± 1.30 —	32	126 ± 3 (11)	9.71 ± 2.0 —	+43	1.23
,,	126 ± 4 (10)	6.78 ± 1.30 —	500 (Acetate)	126 ± 4 (10)	6.73 ± 1.40 —	+ 0	—
146	126 ± 3 (9)	4.90 ± 0.15 —	32	130 ± 5 (8)	6.34 ± 0.28 —	+29	4.24
,,	126 ± 3 (9)	4.90 ± 0.15 —	500	127 ± 3 (10)	7.09 ± 0.28 —	+45	6.76
182	207 ± 6 (12)	2.98 ± 0.33 —	200 (Acetate)	210 ± 8 (12)	4.16 ± 0.24 —	+37	2.22
,,	207 ± 6 (12)	2.98 ± 0.33 —	1000 (Acetate)	199 ± 8 (12)	4.51 ± 0.51 —	+51	2.40
280	135 ± 8 (10)	5.81 ± 0.37 —	32	131 ± 6 (10)	8.03 ± 0.39 —	+38	4.11
,,	127 ± 3 (11)	5.22 ± 0.51 —	20	128 ± 3 (11)	8.81 ± 0.81 —	+69	3.49
,,	131 ± 3 (11)	5.12 ± 0.82 —	20	132 ± 3 (11)	8.99 ± 0.51 —	+76	4.05
,,	131 ± 3 (11)	5.12 ± 0.82 —	10	134 ± 2 (10)	9.90 ± 0.93 —	+93	3.72
,,	131 ± 3 (11)	5.12 ± 0.82 —	1	125 ± 2 (10)	5.99 ± 0.77 —	+17	0.84

containing radiopotassium (half life 12.8 hours)[‡] in the amount of 20 to 280 μg per gram of body weight was injected subcutaneously. The dose of potassium chloride was contained in 2 cc of solution. Urine was collected for 6 hours starting from the time of administration of the potassium chloride solution. The urine was dried and the concentration of radiopotassium in the residue determined as described previously for radiosodium.[1]

The amount of radiopotassium excreted in the urine during the six-hour period was related to the total radiopotassium administered and expressed in per cent. This was done for the results obtained on the experimental animals, as well as for the results obtained on oil injected controls run simultaneously. The significance of the difference in mean percentage excretion of radiopotassium was calculated.

Results. The results of the administration of varying amounts or hormone and potassium chloride to the adrenalectomized male rats are presented in Table I. When 20 μg of potassium chloride per gram of body weight was administered, no significant increment in potassium excretion was found for either 32 μg of desoxycorticosterone or 500 μg of the acetate. In the remaining experiments potassium chloride in the amounts of between 146 to 280 μg per gram of body weight was administered. In each experiment statistically significant increases in potassium excretion were observed. Thus, in one experiment when as little as 10 μg of the steroid was administered, an increase in potassium excretion of 93% was found. The t value was 3.72 indicating a P value of less than 0.01.

Conclusion. Thus far only desoxycorticosterone has been studied with respect to potassium metabolism in the adrenalectomized animal. Under similar conditions it has been previously demonstrated[1] that as little as one microgram produces a significant retention of sodium in the adrenalectomized rat. With radiopotassium 10 micrograms can be detecfluorocardiogram of the border of the right ventricle, and a pressure tracing from the right ventricle are recorded. The fluorocardiogram presents a small presystolic negative wave (transmitted from the auricle); a deep *systolic collapse* (ventricular contraction); and a small, early-diastolic rebound. The pressure tracing presents no waves in diastole. Ventricular systole is accompanied by a *systolic plateau* with a higher initial peak.

[‡] The radiopotassium was supplied by the Monsanto Chemical Co., through the U. S. Atomic Energy Commission.

[1] Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinology*, 1947, 41, 464.

The temporal relationship between the negative *ventricular wave* of the fluorocardiogram and the *systolic plateau* of the pressure tracing is precisely the same, allowing for a brief lag because of the fact that the ventricular wave of the fluorocardiogram starts with the beginning of ejection.

The tracing, recorded by means of the conventional catheter, presents a more rounded curve of intraventricular pressure (Fig. 2B).

Discussion. This study reveals a precise coincidence between the auricular and ventricular waves of the pressure tracing on the one hand and the fluorocardiographic waves on the other hand. The *positive wave* in the pressure tracing is due to the rise in pressure on contraction of the chamber while the *negative wave* in the fluorocardiogram is due to simultaneous decrease in volume of the chamber. Since the dog's heart shifts horizontally only very slightly within the chest, being more vertical than the human heart, experimental fluorocardiograms are nearly pure volume tracings. Thus a further proof is given of the accuracy of fluorocardiography in recording volume changes of the heart whenever the complicating elements of motion are absent or minimal.

Summary. Simultaneous fluorocardiograms of the right auricle and ventricle and tracings of intracardiac pressure reveal a precise temporal coincidence between the waves of the two records. Therefore, a further proof is given of the accuracy of fluorocardiography.

17050 P

Use of Radiopotassium for Detection of Minute Amounts of Desoxycorticosterone.*

RALPH I. DORFMAN. (With the technical assistance of Jack Murphy, Richard Hohman, and Adeline S. Dorfman.)

From the Department of Biochemistry and Medicine, Western Reserve University School of Medicine and Lakeside Hospital, Cleveland, Ohio.

This communication is a report of a preliminary study on the use of radiopotassium for assay of adrenal cortical hormones. The fact that adrenalectomized rats tend to retain potassium, which may be reversed by the administration of adrenal cortical extracts or pure adrenal cortical steroids, is well known. With the use of radiopotassium as little as 10 μg of desoxycorticosterone may be detected in the adrenalectomized rat.

Animals, methods, materials. Albino rats obtained from Carworth Farms, Inc. were used for these studies. The animals were bilaterally adrenalectomized in one stage under ether anesthesia. Experiments were run 24 hours after adrenalectomy. The diet both before and after the operation consisted of Purina Fox chow without added sodium chloride.

The procedure consisted in the subcutaneous administration of the test material, desoxycorticosterone or desoxycorticosterone acetate,† contained in corn oil. The volume of oil was 0.25 cc. One hour after the administration of the steroid, potassium chloride

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* Supported in part by grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, and from Sharp and Dohme, Inc.

† The desoxycorticosterone and desoxycorticosterone acetate were kindly supplied by Ciba Pharmaceutical Products, Inc.

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Conclusion. Thus far only desoxycorticosterone has been studied with respect to potassium metabolism in the adrenalectomized animal. Under similar conditions it has been previously demonstrated[1] that as little as one microgram produces a significant retention of sodium in the adrenalectomized rat. With radiopotassium 10 micrograms can be detected. This method affords a convenient way to evaluate the relative activity of various adrenal cortical steroids and adrenotrophic hormone. Further studies are needed to define the method as a quantitative assay for adrenal cortical steroids.

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[1] Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinology*, 1947, 41, 464.

Summary. By the use of radiopotassium in the adrenalectomized male rat, it is possible to detect as little as 10 μg of desoxycorticosterone. The method affords a convenient means of evaluating the relative activities of adrenal cortical steroids, as well as the influence of adrenotrophic hormone on potassium metabolism.

17051

Time of Appearance of Antibodies to Brain in the Human Receiving Anti-Rabies Vaccine.

R. C. KIRK AND E. E. ECKER.

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Recent reports[1-12] have shown that a disseminated type of encephalomyelitis can be produced in experimental animals by the injection of homologous and heterologous brain tissue, and that the lesions produced resemble the lesions found in the human demyelinating diseases including the encephalomyelitis following anti-rabies vaccination.

This has suggested the possibility that isoimmunization to brain tissue may be the mechanism responsible for the production of these diseases, and, in particular, of the encephalomyelitis following anti-rabies vaccination, since in the latter, the injection of heterologous brain tissue duplicates in many ways the actual experimental procedure which has been followed with the experimental animals.

It seemed of interest, therefore, to determine, in a preliminary study (of 5 cases) whether or not patients receiving anti-rabies vaccine actually develop antibrain antibodies in their sera. The procedure followed consisted of (1) the immunization of rabbits to determine the antigenicity of the brain extract used, and (2) the titration of the sera of patients receiving the anti-rabies vaccine, with this particular antigen.

Materials and methods. I. Preparation of antigens (Lewis[13]):

A. Suspension. The white matter from fresh human brain was dissected free of cortex, connective tissue and blood vessels, cut into thin slices and washed in running water for 30 minutes. It was then ground in a Waring blendor and made into a 30% saline suspension with 0.5% phenol added. This stock solution was used in the immunization of 4 rabbits.

B. Alcoholic extract. The same procedure

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[1] Rivers, T. M., Sprunt, D. H., Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.

[2] Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559.

[3] Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, **61**, 689.

[4] Ferraro, A., and Jervis, G. A., *Arch. Neurol. and Psychiat.*, 1940, **43**, 195.

[5] Ferraro, A., *Arch. Neurol. and Psychiat.*, 1944, **52**, 443.

[6] Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1944, **48**, 297.

[7] Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

[8] Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

[9] Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, **57**, 229.

[10] Morrison, L. Raymond, *Arch. Neurol. and Psychiat.*, 1947 (Oct.), **58**, 391.

[11] Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1948, **88**, 417.

[12] Wolf, A., Kabat, E. A., and Bezer, A. E., *J. Neuropath. and Exp. Neurol.*, 1948, **6**, 333.

[13] Lewis, J. H., *J. Immunol.*, 1933, **24**, 193.

TABLE I.
Complement Fixation Titers Obtained in Rabbits Given Intravenous Injections of Human Brain Mixed with Normal Rabbit Serum.

Rabbit No.	Highest titers obtained			
	8 days	16 days	24 days (Inj. dis.)	40 days
889	1/20	1/40	1/45	1/10
979	1/10	1/30	1/40	1/10
991	1/5	1/20	1/40	1/20
987	1/10	1/30	1/30	1/20

was followed except that after grinding the brain tissue in the Waring blendor, the material was mixed with 10 times its volume of 95% ethyl alcohol and kept in the incubator at 37°C for at least 2 weeks.

II. Immunization of animals (Lewis). The rabbits were given intravenous injections of 1.5 ml of the 1:30 brain suspension every 4 days for 24 days. The suspension was mixed with an equal volume of fresh normal rabbit serum, incubated 2-3 hours at 37°C, and the mixture injected very slowly. The rabbits were bled once every 8 days from the marginal ear vein, the blood allowed to clot, the serum separated by centrifugation and frozen at –30°C until used.

III. Technic of complement fixation. The alcoholic extract alone was used as the antigen, both in the case of the rabbit sera, and in the subsequent fixation with human sera. It was found that a 1:40 dilution of the alcoholic extract in 0.9% saline served adequately without being hemolytic or anticomplementary, and without showing non-specific complement fixation with normal human sera. The amount of antigen per dry weight and in aliquot samples of the extract was checked periodically, and was found to remain relatively constant at 3 mg of solids per ml of the extract. In all instances, 0.1 ml of the 1:40 dilution of the alcoholic extract was used as the antigen; 0.1 ml of the inactivated serum, as well as of each dilution, and two units of guinea pig complement were added and the tubes kept in the refrigerator at 3-4°C overnight. The tubes were then allowed to stand at room temperature for one hour; 1.0 ml of a 2.5% suspension of sensitized sheep red cells added, and the tubes incubated at 37°C for 30 minutes. The results were recorded as 0, 1, 2, 3, and 4 plus fixation. The usual controls (antigen, antiserum, normal serum) were set up at each titration. In addition, the antigen was periodically checked against both human and rabbit positive sera.

IV. Collection of sera from patients. 10-15 ml of blood was withdrawn from patients receiving the Semple anti-rabies vaccine at the beginning of treatment, and, as nearly as possible, every two to three days thereafter. The blood was allowed to clot, the serum separated by centrifugation, and frozen at –30°C until used.

Experimental. I. Four rabbits, each weighing about 5 lbs, were given 6 intravenous injections of 3 ml of the human brain suspension mixed with normal rabbit serum at 4-day intervals as described. Control sera obtained at the start of the injections showed a + fixation of complement when 0.1 ml of the undiluted serum was used, but no fixation was observed in a 1:5 or higher dilution. The subsequent titers obtained are shown in the accompanying table.[1] It is to be noted that the injections were discontinued on the 24th day. The sera obtained from these animals were used as controls in the subsequent fixation tests with human sera.

II. The sera from the 5 patients receiving the anti-rabies vaccine were then titrated in a similar manner. The results obtained are shown in Fig. 1 and 2.

Discussion of cases. Cases No. 1, 3, and 4. These patients were given the routine 14 daily injections of the Semple anti-rabies vaccine (25% suspension phenol-killed rabbit brain virus) following a dog bite in which the dog was not found for examination. They tolerated the injections well and showed very

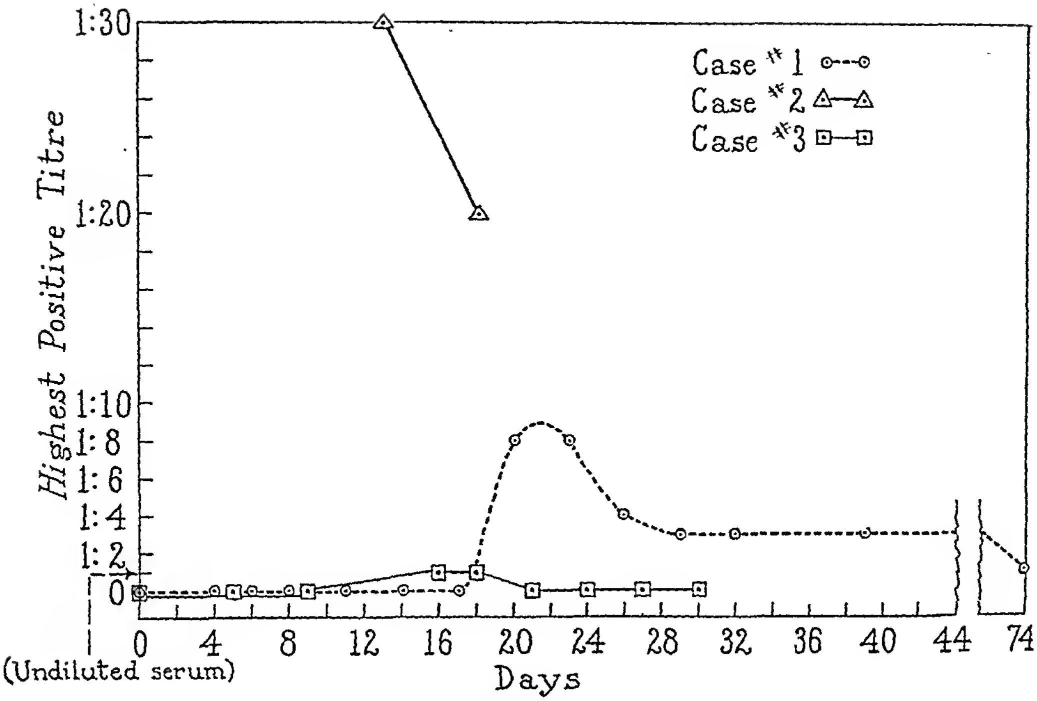


Fig. 1.
Complement fixation titers in cases 1, 2, and 3.

little reaction. Cases 3 and 4 developed some swelling and redness at the site of the injections between the 10th and 14th dose. In each case, the sera gave a negative complement fixation reaction until the 15th to 18th day at which time antibodies to the brain antigen appeared. (See Fig. 1 and 2).

Case No. 2. This patient was a 9-year-old Italian boy who was bitten by a dog on August 17, 1948. The dog was not found and for this reason injections of the antirabies vaccine were started on August 23, 1948. He received a total of 5 injections, each of which produced considerable swelling and redness at the site of injection. Because of the discomfort, the patient and his parents objected strenuously to the treatment, and the patient was discharged against medical advice. He had given no previous history of allergy, and his initial physical examination was entirely normal. On August 29, 1948, 6 days after the first injection with the antirabies vaccine, the patient became lethargic, vomited, developed a fever, anorexia, and pain in the right upper abdominal quadrant. He was readmitted to the hospital where his temperature was found to be 39.2°C, pulse 120, and his appearance lethargic. He appeared "quite ill." Examination revealed a mild inflammation of the nasopharynx, a stiff neck, and a positive Kernig and Brudzinski. The total white count on admission was 9,800 with a slight leukocytosis. The spinal fluid showed 23 cells, of which 18 were described as monocytes and 5 as polymorphonuclear leukocytes. The CSF protein was 70, sugar 100, and chlorides (as NaCl) 734.

It was possible to obtain only 2 samples of his blood for titration, one on September 7th, 9 days following the development of symptoms, and the other 3 days later. Both samples showed a high titer of complement fixation when compared to the other cases studied, the second blood sample showed a falling titer. Apparently, the antibody titer had begun considerably earlier than in the other four cases, and it may well have reached an even higher concentration than that found in the sample of September 7th. The patient's symptoms subsided and he was dis-

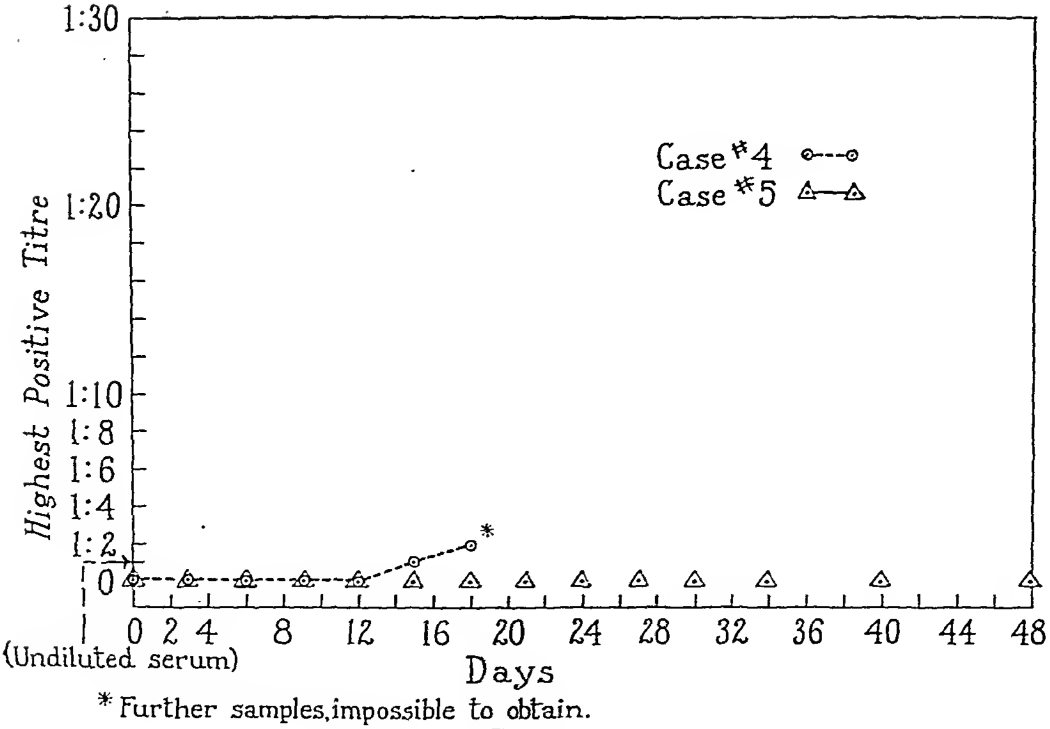
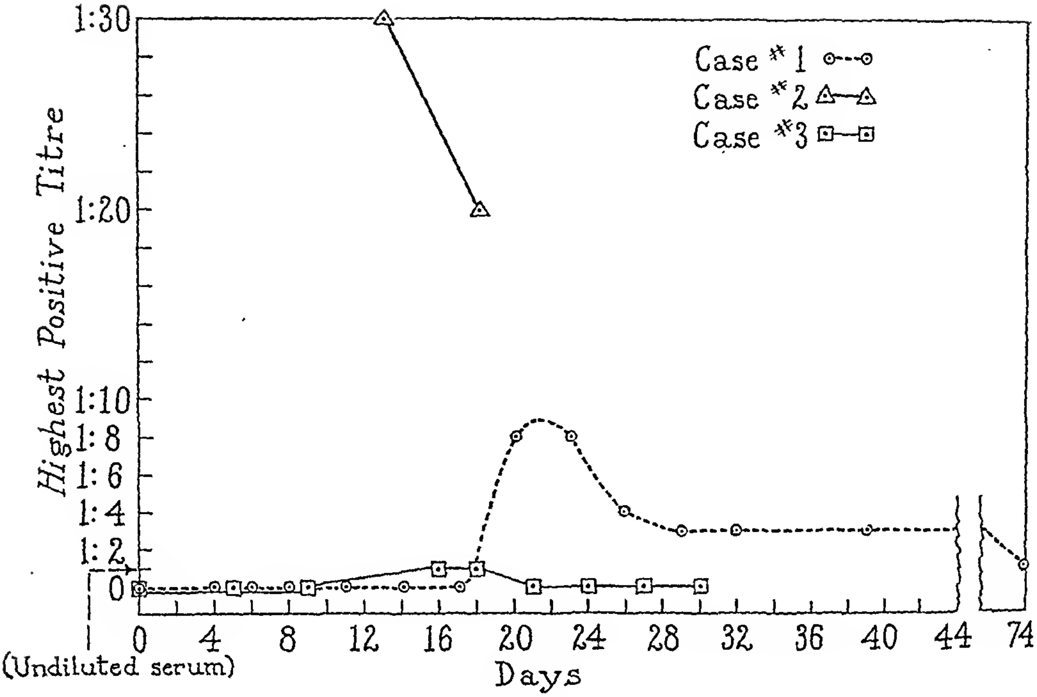


FIG. 2.
Complement fixation titers in cases 4 and 5.

charged entirely well on his 14th hospital day, with the provisional diagnosis of "possible encephalitis following anti-rabies treatment."

Case No. 5. This 34-year-old white female was bitten on the face by a stray dog. She was given the routine 14 daily injections of the vaccine and showed only a slight amount of redness and swelling at the site of injections between the 10th and 14th day of treatment. Blood samples obtained up to the 60th day after the beginning of treatment failed to show fixation of complement. The tests on the sera of this patient were repeated with varying dilutions of the antigen from 1 to 20 up to 1 to 1280, but no fixation occurred. It was then thought that the patient's sera might contain an inhibiting substance. In order to determine this, portions of these same sera (0.3 ml) were mixed with equal portions of the fresh positive serum of Case No. 2. This mixture was inactivated at 55°C for 30 minutes and varying dilutions were then used in fixation reactions with the brain antigen as previously described. Controls of the known positive serum diluted in the same manner with a normal serum, and of the positive serum alone were run at the same time. No inhibition of complement fixation was found to occur with the sera of case No. 5 or with the controls. It was therefore thought that patient No. 5 failed to show antibrain antibodies following treatment with antirabies vaccine.

Comment. The present work confirms that of Lewis in showing that antibodies to brain tissue are produced in the rabbit by the intravenous injection of foreign brain substance. It also provides evidence that antibodies to human brain are produced in patients receiving the Semple antirabies vaccine. Case 2 suggests the possibility that a markedly increased antibrain antibody titer may occur in those patients developing an encephalitis following this treatment.



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Complement fixation titers in cases 1, 2, and 3.

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TABLE I.
In vitro Effect of Tuberculin on Blood Leucocytes in the Presence of Normal and Tuberculous Plasma.

Normal cells + Normal plasma[*]	0.4[†]	0.4						
Normal cells + Tuberculous plasma			0.4	0.4				
Tuberculous cells + Normal plasma					0.4	0.4		
Tuberculous cells + Tuberculous plasma							0.4	0.4
Tuberculin antigen	0.1		0.1		0.1		0.1	
Saline		0.1		0.1		0.1		0.1
Total WBC								
5 min.	7,760	7,440	7,810	8,070	11,500	12,150	5,070	4,000
60 min.	7,790	7,450	5,980	8,130	11,466	12,220	3,870	4,050
% decrement	+0.5	+0.3	—23.2	+0.8	—1.0	+0.5	—23.8	+1.2

[*] Normal cells and normal plasma are obtained from a healthy tuberculin-negative subject.
[†] *Amounts refer to cubic centimeters.*

adjusted so that final cell concentrations varied between 4,000 - 15,000 cells per cu mm. To 0.4 cc of such white cell suspensions was added 0.1 cc of tuberculin antigen (prepared as described earlier[11]). As cell system controls, 0.1 cc of isotonic saline was added to a duplicate series of cell suspensions. The test tubes used in these experiments have been coated with organosilicone to diminish nonspecific cytolysis. White blood counts were done with a mechanical pipette filler and calibrated pipettes (15 squares on 2 chambers of the standard hemocytometer slide were counted). Cytolysis was demonstrated by doing total white counts before and after a 60 minute period of incubation at 37°C.

Results. A sample protocol illustrates the results obtained in typical experiments.

1. White cells from normal tuberculin-negative humans as well as from tuberculous patients can be lysed when suspended in the plasma of tuberculous patients in the presence of old tuberculin.
2. Adequately washed tuberculous cells will show no cytolysis by tuberculin when such cells are suspended in normal plasma.
3. Such cytolysis by tuberculin varies between 20-35% of the total cells present under conditions of these experiments.
4. When isotonic saline is substituted for the tuberculin antigen, no cytolysis beyond 2.2% could be demonstrated. This variation in cell count is felt to be the amount of error inherent in the method used.

Discussion. From the foregoing, it seems apparent that tuberculin antigen can exert a cytotoxic effect on washed normal tuberculin-negative human white cells as well as on those of tuberculous patients provided tuberculous plasma (or serum) is present in the system. Results of an earlier report[10] in which tuberculous cells were similarly lysed in normal serum were probably due to the presence of traces of tuberculous plasma on the surfaces of unwashed or insufficiently washed white cells. Since it has been shown[11] that normal tuberculin-negative human white cells have the same capacity to adsorb tuberculin onto their cell surfaces as do tuberculous cells, it would appear that the vital factor in cytolysis of white cells by tuberculin is some component in tuberculous plasma. Further experiments to elucidate the nature and origin of this plasma factor are now in progress.

Summary. Under the conditions of the experiments reported here, the cytolysis of human white blood cells by tuberculin occurs

17052

A Plasma Factor Responsible for *in vitro* Lysis of Leucocytes by Tuberculoprotein.*

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Rich and Lewis,[1] studying living tissue cultures of washed leucocytes, showed that tuberculin in proper concentration had a selective toxic effect on cells from tuberculous animals. Since this phenomenon occurred even when allergic cells were suspended in the plasma of a normal animal, it was felt that this manifestation of tuberculin sensitivity was not dependent on circulating plasma antibodies of the sensitized animal but rather was a property inherent in the cells. Subsequent tissue culture studies[2-5] have confirmed this specific cytotoxic effect of tuberculin on leucocytes from tuberculous animals and again the reaction has been attributed to a property of the cells rather than to any serum antibody. Chase[6] and others[7,8] demonstrated that the tuberculin type of hypersensitivity could be transferred passively to normal guinea pigs by injection of the cells of peritoneal exudates, lymph nodes or spleens of guinea pigs sensitized to tuberculin by the injection of heat killed tubercle bacilli, thus confirming some crucial property of cells as the basis of delayed, tuberculin-type hypersensitivity.

More recently, it has been shown[9] that bacterial products of the tubercle bacillus exert a significant lytic effect *in vitro* on leucocytes from tuberculous animals after a one-hour period of incubation. A similar cytotoxic response has been observed on white cells from tuberculous humans.[10] Since these human white cells were suspended in normal serum (or plasma), it was concluded that the cytotoxic action of tuberculin was specific to tuberculous-type white cells and not dependent on any serum component.

It is now apparent that if such tuberculous-type white cells are thoroughly washed and then suspended in normal plasma, no lytic effect by tuberculin can be demonstrated over the one hour period of incubation. But if such washed cells, or even white cells from a normal tuberculin-negative human, are added to the plasma of a tuberculous human in the presence of old tuberculin, white cell lysis occurs during the course of one hour. It is the purpose of this report to demonstrate that a factor in tuberculous plasma is necessary for the lysis of white cells by tuberculin.

Experimental. Using a method previously described,[11] white cells from normal tuberculin-negative humans and from tuberculous patients hospitalized for acute tuberculous infection were concentrated and thoroughly washed with isotonic saline solution. The resulting cell concentrates were then suspended in normal human plasma as well as in tuberculous plasma, the amount of plasma being

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† Research Fellow, National Institute of Health.

[1] Rich, A. R., and Lewis, M. R., PROC. SOC. EXP. BIOL. AND MED., 1927-28, **25**, 596.

[2] Aronson, J. D., *J. Exp. Med.*, 1931, **54**, 387.

[3] Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

[4] Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

[5] Heilman, D. H., Feldman, W. H., and Mann, F. C., *Am. Rev. Tub.*, 1944, **50**, 344.

[6] Chase, M., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

[7] Cummings, M. M., Hoyt, M., and Gottshall, R. Y., *Public Health Rep.*, 1947, **62**, 994.

[8] Stavitsky, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 225.

[9] Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 269.

[10] Fremont-Smith, P., and Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 502.

[11] Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 369.

TABLE I.
Preparation and Treatment of Animals.

Group	No. of animals	Preparation of animals	Treatment
I	15	None	None
II	20	Anesthetic (Nembutal) Surgical mobilization of spleen	”
III	20	Anesthetic (Nembutal) Surgical mobilization of spleen	600 r total body X irradiation inclusive of spleen
IV	20	Anesthetic (Nembutal) Surgical mobilization of spleen	600 r total body X irradiation exclusive of spleen

1-3). In Group III (spleens unprotected), however, these values were markedly reduced between the sixth and eighteenth day after irradiation. The mean reticulocyte value of Group III animals (spleens unprotected) was reduced to less than 0.1% by 2 days and remained reduced through nine days (Fig. 4). The mean reticulocyte value of Group IV mice (lead-protected spleens) was not significantly reduced at any time; a definite increase above the normal value occurred between the third and fourteenth day. The mean platelet value of Group III (spleens unprotected) fell gradually to a minimum of 15,000 cu mm on the eighth day and rose to a normal value by the eighteenth day after irradiation, whereas the platelet value of Group IV (lead-protected spleens) reached a minimum of 230,000 cu mm on the ninth day and rose to a normal value by the eleventh day. (Fig. 5) The mean leucocyte value of Group III (spleens unpro-

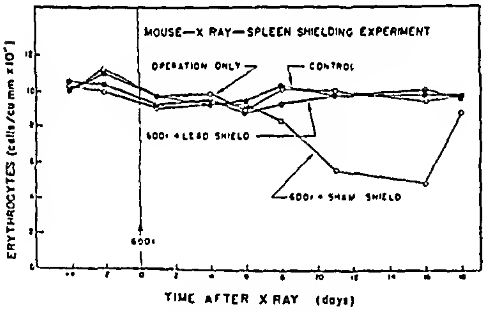

FIG. 2.
The erythrocyte values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

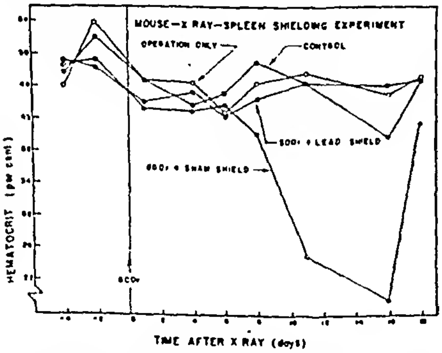

FIG. 3.
The hematocrit values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

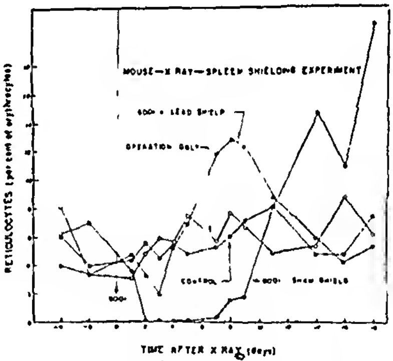

FIG. 4.
The reticulocyte values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

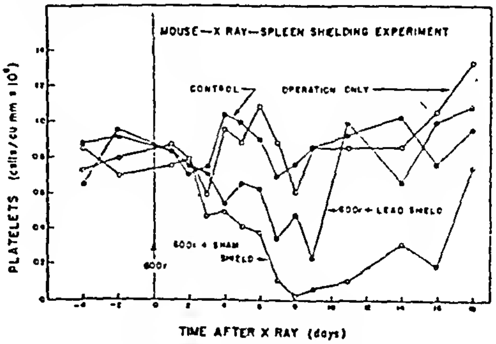

FIG. 5.
The platelet values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

only in the presence of plasma from tuberculous subjects. White blood cells from healthy tuberculin-negative humans will undergo similar tuberculin cytolysis in the presence of such tuberculous plasma.

17053

The Role of the Spleen in Radiation Injury.[*]

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Ectopic blood formation in the spleens of mice injected with a dose of 2.0 microcuries per gram of body weight of radiostrontium (Sr^{89}), as shown by Jacobson *et al.*,[1,2] was sufficient to obviate the development of anemia even though the bone marrow was largely destroyed and only gradually reconstituted over a period in excess of 100 days. Splenectomized mice given this dose developed a severe anemia, recovery from which occurred only as the hematopoietic activity of the bone marrow recovered. This communication describes a somewhat different but related technic for studying the significance of the spleen in recovery from or compensation for radiation injury.

Materials and Methods. Four groups of young female mice were prepared as indicated in Table I. Mice in Group I were untreated controls. The mice in Groups II, III, and IV were anesthetized, an incision made in the left upper quadrant of the abdomen, and the spleen brought out through the abdominal incision with the main pedicle intact. Group III and Group IV mice were irradiated with 600 r whole-body X radiation (250 Kv) except that during the irradiation the mobilized spleens of Group IV mice were placed in one-quarter inch thick lead boxes with openings for the pedicle only. The mobilized spleens of Group III mice were placed in thin paraffin boxes which offered no appreciable shielding from the radiation. The mobilized spleens of Group II mice (operated controls) were placed in lead boxes for a period equal to the time that Group III and Group IV spleens were thus contained. The radiation required approximately 12 minutes after which the spleens of groups II, III, and IV mice were returned to the abdominal cavity and the operative incisions sutured.

Results. Hematologic studies were made on all 4 groups. Animals from each group were sacrificed at intervals for histopathologic study.

The mean hemoglobin, erythrocyte, and hematocrit values of Group IV (lead-protected spleens) were not significantly altered when compared to control Groups I and II (Fig.

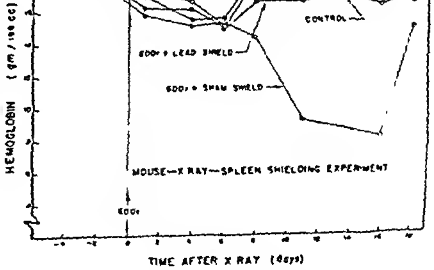

FIG. 1.
The hemoglobin values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

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[*] Aided in part by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council and a grant from Armour and Company.

[1] Jacobson, L. O., and Simmons, E. L., *Anat. Rec.*, 1948, **100**, abstract.

[2] Jacobson, L. O., Simmons, E. L., and Block, M. H., National Nuclear Energy Series, Div. IV, Vol. 22B.

17054

Proteolytic Activity of Hemophilic Plasma.

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Tagnon, Davidson, and Taylor[1] reported that the proteolytic activity of hemophilic plasma was lower than normal, but subsequently this group of investigators found no such difference.[2] In both studies the proteolytic enzyme was activated by treatment with chloroform; proteolytic activity was then measured by the rate of destruction of fibrinogen and fibrin[1] or by the rate of digestion of casein.[2]

Human blood plasma contains the inactive proteolytic enzyme precursor, plasminogen,*[3] which can be activated partially at least by chloroform treatment; activation with streptokinase appears to be much more effective. Plasma also contains an antiproteolytic factor[7-9] that must be removed or destroyed in order to obtain a true measure of the enzyme activity. In the procedure herein described the plasminogen has been separated from the antiproteolytic material by an alcohol fractionation similar to that devised by Cohn *et al.*[10,11] for the separation of plasma proteins. The enzyme was then activated with streptokinase,[12] and the plasmin activity was estimated from a measurement of fibrinogenolysis time.[1,13] A comparison of hemophilic and normal plasmas showed little if any difference in plasminogen content.

Experimental. Reagents. 1. 50% alcohol. 500 ml of 95% ethanol were diluted with distilled water to 950 ml.

2. Acetate buffer, pH 4. 12 ml of 1 M sodium acetate and 72 ml of 1 M acetic acid were mixed and diluted to 1 liter with distilled water.

3. Phosphate buffer, pH 10.1, with sodium citrate. 13.4 g of $Na_2HPO_4 \cdot 7H_2O$ and 29.4 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ were dissolved in approximately 500 ml distilled water. Normal sodium hydroxide was added to adjust the pH to 10.1, and the solution was then diluted to 1 liter.

4. Phosphate buffer, pH 7.2, with sodium citrate and sodium chloride. 4.14 g of $NaH_2PO_4 \cdot H_2O$, 8.82 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ and 8.91 g of NaCl were dissolved in approximately 500 ml distilled water. The pH of the solution was adjusted to 7.2 with normal sodium hydroxide, and the solution was then diluted to 1 liter.

5. Streptokinase. A crude preparation of this enzyme was prepared from a β-hemolytic streptococcus medium by the alcohol precipitation method of Garner and Tillett.[14] The precipitate was dried from the frozen state

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[1] Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1943, **22**, 127.

[2] Lewis, J. H., Davidson, C. S., Minot, G. R., Soulier, J. P., Tagnon, H. J., and Taylor, F. H. L., *J. Clin. Invest.*, 1946, **25**, 870.

* Several proposals for the nomenclature of the active enzyme and its precursor have been made. They are a) plasmin and plasminogen;[3] b) fibrinolysin and profibrinolysin;[4] c) serum tryptase and tryptogen;[5] d) serum protease and lytic factor[6] respectively.

[3] Christensen, L. R., and MacLeod. C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

[4] Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

[5] Ferguson, J. H., *Science*, 1947, **105**, 488.

[6] Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

[7] Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.

[8] Grob, D., *J. Gen. Physiology*, 1943, **26**, 405.

[9] Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 337.

[10] Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

[11] Edsall, J. T., *Advances in Protein Chemistry*, 1947, **3**, 383.

[12] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

[13] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

[14] Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

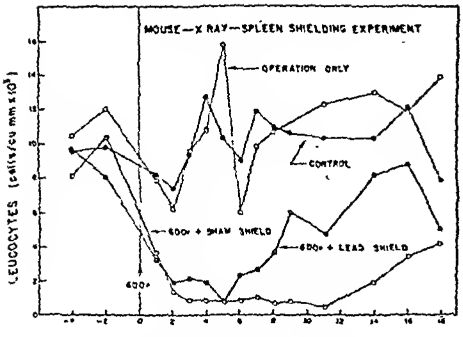

FIG. 6.

The leucocyte values of control mice and mice exposed to 600 r with and without lead protection of the spleen.

tected) was reduced below 1000 per cu mm by the third day and remained below 1000 through the eleventh day after irradiation. The mean leucocyte value of Group IV (lead-protected spleens) fell to a minimum of circa 2000 per cu mm only and rose to a relatively normal value by the ninth day after irradiation (Fig. 6).

The spleens of Group III (spleens unprotected) decreased markedly in size within twenty-four hours and remained thus reduced in size beyond the tenth day after irradiation. The spleens of Group IV (lead-protected spleens) increased in size reaching in some instances approximately twice the size of controls by the third day.

The histologic studies revealed that hematopoietic tissue in Group III animals that received 600 r inclusive of the spleen was largely destroyed with significant regeneration only beginning after the sixth day. A comparable degree of destruction of hematopoietic tissue occurred in Group IV animals except in the lead-protected spleens where a marked increase in erythrocytopoiesis, megakaryocytopoiesis, and granulocytopoiesis was already apparent by 18 hours after exposure. This ectopic blood formation in the spleen increased rapidly in extent. Lymphatic tissue in these lead-protected spleens decreased however, as the erythro-, granulo-, and megakaryocytopoiesis increased. By forty-eight hours after irradiation the amount of lymphatic tissue remaining in the lead-protected spleens was approximately 75% less than controls and consisted largely of medium and small lymphocytes about the arterioles in the white pulp.

Summary and conclusions. These hematologic and histologic data indicate that:

1) Severe anemia, leucopenia, and thrombocytopenia develop in mice after a single dose of 600 r whole-body X radiation.

2) Ectopic erythrocytopoiesis, in the lead-protected spleens of mice given 600 r whole-body X radiation (exclusive of spleens) compensates with such rapidity and so extensively for the destruction and interruption of this activity in the marrow spaces that no anemia of significance becomes apparent. Ectopic granulocytopoiesis and megakaryocytopoiesis in the lead-protected spleens compensates significantly but at a slower pace and less completely for the bone marrow destruction.

3) A marked and sustained decrease in the amount of lymphatic tissue is produced in the lead-protected spleens of animals given 600 r whole-body X radiation. This decrease in lymphatic tissue may perhaps be a result of (a) unsuccessful competition of the lymphatic tissue with the ectopic hematopoiesis for nutritional requirements, (b) actual indirect effect of radiation and (c) a differential humoral suppression from some unknown site.

The rapidity with which erythrocytopoiesis transfers from the X-ray damaged bone marrow to the lead-protected spleen in the absence of anemia suggests that the mechanism of stimulation of erythrocytopoiesis under the conditions of this experiment may involve some factor or factors other than, or in addition to, the accepted hemoglobin-oxygen relationship.

This technic permits more or less exclusive protection of the spleen or the appendix or other visceral tissues from irradiation while applying various dosages to the remainder of the body. It provides a method of studying potential sites and mechanism of the production of ectopic blood formation, possible secondary effects of radiation as well as offering possibilities for determining the potential role of such sites in immune reactions, in preventing or alleviating radiation-induced hemorrhagic phenomena and in the study of survival or recovery from radiation injury.

rate in the buffer reagents the fibrinogen did not clot until thrombin was added.

Activation and Test for Proteolytic Activity. 5 ml of the solution to be tested were placed in a constant temperature bath at 38°C for 5 minutes; 0.2 ml of the streptokinase suspension was then added. Aliquots of 0.2 ml were removed at one-minute intervals following the streptokinase addition, and these were immediately tested for clotting power by the addition of 0.2 ml of thrombin solution. The time at which thrombin no longer clotted the test solution was recorded as the fibrinogenolysis time. This was the time required for the streptokinase to activate the enzyme and for the latter to digest the fibrinogen substrate to the point where it no longer coagulated.

Results. Table I shows the fibrinogenolytic activities of 3 hemophilic plasmas and of 5 normal human plasmas.

The fraction obtained from hemophilic patient No. 3 showed no activity when the 100% solution was treated with 25 mg of streptokinase. However, good proteolytic activity was demonstrated in this plasma when the relative proportion of streptokinase to plasma was greatly increased. This suggests that the fraction from this plasma contained a high concentration of antistreptokinase[16] which could be overcome by treatment with large amounts of streptokinase.

Discussion. By the precipitation of plasminogen from plasma under the conditions described, its activation by streptokinase and its proteolytic activity could de demonstrated without interference from the antiproteolytic factor. When whole plasma was treated with streptokinase in the same proportion as was used with fractionated plasma, this rapid digestion of fibrinogen was not observed. Neither could a solution of the first alcohol precipitate of plasma be rapidly activated by streptokinase.

Christensen and MacLeod[3] and Kaplan[17] have shown that the chloroform activated and the streptokinase activated proteolytic enzymes are identical. Since the streptokinase activated proteolytic activity of hemophilic plasma in this experiment was found to be comparable to that of normal human plasma, the results confirm the conclusions of Lewis, *et al.*,[2] that the proteolytic activity of hemophilic plasmas is not impaired.

Summary. 1. A method is described by which the proteolytic activity of human normal and hemophilic plasmas may be compared.

2. The proteolytic activity of 3 hemophilic plasmas was found to be comparable to that of normal plasmas.

Gratitude is expressed to Dr. Tyree C. Wyatt and Dr. John Houpis of the Pediatrics Departments, Syracuse University College of Medicine and Syracuse Memorial Hospital for their cooperation in securing the bloods from the hemophilic patients.

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[16] Kaplan, M. H., in collaboration with the Commission on Acute Respiratory Diseases, *J. Clin. Invest.*, 1946, **25**, 347.

[17] Kaplan, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 1940.

TABLE I.
Fibrinogenolytic Activity of the Plasmin Fraction of Normal and Hemolytic Plasmas Activated with Streptokinase.

			Fibrinogenolysis time		
			% conc. of plasma		
	Subject	Amt of streptokinase added to 5 ml of solution (mg)	100 Min.	50 Min.	12.5 Min.
Normal	1	25	4		21
Plasma	2	25	5		15
Preparations	3	25	4		8
	4	25	8		35
	5	25	4		18
Hemophilic	1	25	7		18
Plasma	2	25	6		19
Preparations	3	25	No act.		41
		75	—	7	—
		200	6		—
Beef fibrinogen		25	Good clot formation after 21 hr		
		200	Good clot formation after 21 hr		

and resuspended in the phosphate buffer just before use.

6. Beef fibrinogen was prepared according to Ware, Guest, and Seegers.[15] A 0.4% solution was made up in the phosphate buffer, pH 7.2. This fibrinogen was free of any plasminogen that could be activated by streptokinase.

7. Thrombin. Sufficient dried Harvard Fraction III-2† was dissolved in 0.85% sodium chloride so that 0.2 ml clotted 0.2 ml of a 0.4% fibrinogen solution in approximately 1 minute.

Plasma Fractionation Procedure. Citrated blood (12 ml whole blood and 1 ml 4% sodium citrate) was centrifuged; 5 ml of the plasma were measured into a 50 ml centrifuge tube and cooled to 1°C in an ice bath. 2.5 ml of cold acetate buffer were added, giving a pH of approximately 6.8. 12.5 ml of the cold ethanol solution were then added slowly from a pipette with constant stirring to give a precipitate containing the fibrinogen, plasminogen, antiproteolytic material and other proteins. After standing in an ice bath for 30 minutes the precipitate was removed by centrifuging in the cold (3-5°C), and the supernatant solution was discarded.

The precipitate was then suspended in 0.3 ml of phosphate buffer, pH 10.1, and the suspension was dissolved by the addition of 5 ml of cold water. To the solution were added 10 ml of cold water and 15 ml of cold ethanol solution. The pH of this medium was about 7.6. It was cooled in an ice bath for 30 minutes, centrifuged in the cold, and the supernatant solution (containing the antiproteolytic material) was discarded. The precipitate was redissolved in phosphate buffer, pH 7.2, and the final volume was adjusted with buffer to 5 ml. The buffer was prewarmed to 38°C, and was added just before the sample was treated with streptokinase.

This solution, containing the original plasma concentration of plasminogen and fibrinogen, was tested at both 100% and 12.5% of the original plasma concentration. In testing the undiluted solution (100%) the fibrinogen *of the original plasma served as the substrate.* In testing at 12.5% concentration, 1 ml of the solution was diluted with 7 ml of the beef fibrinogen substrate. By including cit-

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[15] Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.*, 1947, **13**, 231.

† The fraction III-2 was obtained through the courtesy of Dr. John T. Edsall. It was prepared under a contract between the Office of Scientific Research and Development and Harvard University from human blood collected by the American Red Cross.

TABLE I.
Effect of Boric Acid Injected with Alloxan on Survival and Diabetes.

Diet	No. of rats	Treatment*	Survival (%)		Blood sugar, 48 hr (mg %)
			2 days	7 days	
High lard	10	A	50	10	405 (350-478)
,, ,,	8	A + B	100	88	151 (114-213)
High lard—tocopherol	10	A	60	10	461 (376-643)
,, ,, ,,	4	A + B	100	75	142 (111-135)
Low fat	6	A	100	83	467 (230-898)
,, ,,	6	A + B	100	100	123 (111-136)
Low fat—tocopherol	5	A	100	80	612 (479-873)
,, ,, ,,	5	A + B	100	100	134 (94-200)

* A = Alloxan, 160 mg/kg; B = Boric acid, 160 mg/kg.

comparison of hemolysis caused by alloxan with and without boric acid.

Results. In Table I are given the blood sugar and survival data for rats receiving alloxan and alloxan with boric acid. In every group those animals which received alloxan with boric acid had nearly all normal blood sugars, while all receiving alloxan alone were diabetic. When alloxan was injected survival was much better in the groups receiving the low-fat ration than in those which had the high-fat diet although the diabetes was equally severe in both cases. When boric acid was injected with alloxan there was definite improvement in survival in the groups on the high-lard diet. There was no mortality during the first 2 days following injection, and only 2 animals of the 12 died within 7 days, while with alloxan alone 18 out of 20 had died during this period. All animals on the low-fat diet which had been injected with alloxan and boric acid survived the 7 day period, but with alloxan alone, only 2 of 11 had died. In contrast to the animals which received alloxan alone blood NPN was never elevated in rats treated with the combination of alloxan and boric acid.

We found much less serious effects following a second injection than did Kuhn and Quadbeck. Of the whole series of animals, only 3 died after the second injection, all within the first 2 days. Two had been on the high-lard diet, one of the low-fat diet. None of the survivors showed elevated blood sugar levels and urine sugars determined a week or so after injection were almost all negative, with no more than a trace of sugar found in any sample. In Table II are given values found on animals which received 2 to 4 injections of alloxan with boric acid. In no case was there an elevated sugar, but when alloxan was given a diabetic sugar level was found in those animals which survived for 2 days following the injection.

The primary purpose of Table II is comparison of hemolysis following injection of alloxan with and without boric acid. Hemolysis is never observed in animals receiving tocopherol and is relatively mild in animals on a low-fat diet. Therefore, all of the animals considered here were on the high-lard ration without tocopherol. The first animals observed showed only mild hemoglobinuria, an estimated trace to +, as compared with an almost uniform ++ to ++++ in animals previously observed which had been injected with alloxan alone. However, since the observations were only semi-quantitative and there might be some difference in interpretation and, of greater importance, since we had found that tocopherol intake in the pre-experimental period might have some effect, simultaneous injections of alloxan and alloxan with boric acid were made. Five such pairs were studied, 2 on a first injection, 3 others by injecting one of a pair of animals which had both previously been injected with alloxan plus boric acid with alloxan alone, while the other again received alloxan with boric acid. In every case hemolysis as estimated from hemoglobinemia and hemoglobinuria was ++ or ++++ when alloxan was given

17055

Effect of Boric Acid on Biological Activity of Alloxan.

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In the course of studies on the effect of dietary factors in alloxan diabetes it was found[1] that when rats on a tocopherol deficient diet are injected with alloxan they exhibit marked hemoglobinemia and hemoglobinuria. This reaction has not been observed in animals receiving tocopherol. Because he thought it might be of interest to use in connection with this problem, Professor Richard Kuhn (Heidelberg) kindly sent to us in advance of publication the results of a study by Kuhn and Quadbeck[2] on the effect of simultaneous administration of alloxan and boric acid. These authors found that when boric acid was administered with alloxan, blood sugar values were lower and survival time longer than when alloxan was given alone. When a second injection of alloxan and boric acid was given a week later, however, severe diabetes was produced. Boric acid is known to increase the activity of alloxan in its lactin form[3,4] and they attribute the protective effect of boric acid to increased reactivity of alloxan in other active centers so that less reaches the pancreas; failure of protection on reinjection may indicate saturation or inactivation of these centers. We have carried out experiments similar to those of Kuhn and Quadbeck on animals receiving our special rations, and have studied hemolysis as well as diabetes.

Experimental. The general procedure in this study was the same as that previously reported.[1] Two types of diet were used: a high-lard diet (casein 20%, sugar 36%, lard 38%, cod liver oil 2%, salt mixture 4%) which in the previous experiment had caused marked hemolysis and high mortality, and a low-fat diet (casein 20%, sugar 76%, salt mixture 4%, supplemented with 3 drops of corn oil per day and 3 drops of percomorph oil per week) where hemolysis and mortality were low and there was consequently better opportunity of observing the development of diabetes. All animals received a daily supplement of crystalline B-vitamins and the tocopherol-treated groups received 3 mg of mixed tocopherols* daily. Female rats of the Sprague-Dawley strain weighing 100 to 145 g were used. They were kept on the experimental diet for a month before injection of alloxan.

Alloxan and boric acid (10-16 mg/cc) were injected intraperitoneally at a level of 160 mg/kg each. When both were given they were injected separately but almost simultaneously. Blood sugars were taken the second day after injection and determined by the method of Somogyi[5] as modified by Nelson.[6,†] In some cases blood NPN was also determined. Hemolysis was estimated by hematocrit determinations 15 and 30 minutes after injection and by the amount of hemoglobin observed in the urine which was collected on filter paper.

The study previously reported[1] had furnished a large number of control animals so only a few injections of alloxan alone were made in the present series. These special control animals were used for simultaneous which would reduce the effective concentration of alloxan at any time.

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[1] György, P., and Rose, C. S., *Science*, 1948, **108**, 716.

[2] Kuhn, R., and Quadbeck, G., unpublished.

[3] Kuhn, R., and Weygand, F., *Ber. d. deutsch. chem. Gesellsch.*, 1935, **68**, 1282.

[4] Kuhn, R., Reinemund, K., Weygand, F., and Ströbele, R., *Ber. d. deutsch. chem. Gesellsch.*, 1935, **68**, 1765.

* Kindly furnished by Distillation Products, Inc., Rochester, N. Y.

[5] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 61.

[6] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

† The blood sugar determinations were made in the George S. Cox Institute through the courtesy of Dr. F. D. W. Lukens.

Our results confirmed completely those of Kuhn and Quadbeck with regard to the effect of a first injection of alloxan with boric acid. We are unable to account satisfactorily for the discrepancy between our results and theirs on second injection. They found the second injection much more toxic than the first while we observed this in only a few cases. No effect of alloxan could be seen from most of our blood sugar values, but the mild hemolysis showed that it was not always completely inactivated. A somewhat more serious damage to the pancreas might have occurred in the animals of Kuhn and Quadbeck, not sufficient to cause frank diabetes but leaving them more susceptible to injury by the second dose. This is more probable since their dose of alloxan varied from 150 to 250 mg/kg while ours was always 160 mg/kg.

Summary. When rats on special rations were injected simultaneously with alloxan and boric acid, the deleterious effects of the alloxan were reduced. Only a few animals showed mild diabetes while alloxan alone produced severe diabetes in all cases. Survival for 7 days in animals receiving a high-lard diet was raised from 10% to 80%. Intravascular hemolysis in tocopherol-deficient animals on the high-lard diet, always severe with alloxan alone, was absent or mild when the boric acid was given.

17056

Transfusion of Leukocytes and Products of Disintegrated Leukocytes.*

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It is a common clinical observation that no demonstrable rise in leukocyte count can be obtained despite repeated transfusions of whole blood, even when given to individuals with marked leukopenia. It has been thought that this failure to successfully increase the leukocyte count by transfusion might be due either to the short life span of leukocytes, to their rapid disappearance from stored blood or possibly to type incompatibilities. This investigation was undertaken to determine whether transfused leukocytes would remain in the blood stream, and if not, to study the mechanism of their removal.

Methods. White blood cells were obtained from the rabbit's peritoneal cavity by a modification of the method of Mudd and coworkers.[1] 300 to 500 cc of physiologic saline were injected into the peritoneal cavity in the evening and a similar amount of saline the next morning about 15 hours later. Overdistension of the rabbit's peritoneum is poorly tolerated and may result in death of the animal. Four hours after the second injection of saline, the fluid was removed from the peritoneal cavity through a 16 gauge needle, using 0.5 cc (5 mg) of heparin as an anticoagulant. The fluid obtained was centrifuged at a low speed for 5 minutes and the sediment resuspended in 10 to 20 cc of Tyrode's solution so that the leukocyte count was in the neighborhood of 50,000 cells per cmm. This cell suspension was then administered intravenously to the same rabbit (autotransfusion) or into other rabbits (heterotransfusion). The cell free peritoneal fluid was also administered intravenously. The cells obtained by this method exhibited apparently normal ameboid movement, were actively phagocytic for *staphylococcus albus*, and took up vital stains. About 90 per cent

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* This work was supported in part by a grant from the National Vitamin Foundation under the direction of Dr. A. D. Welch.

[1] Mudd, S., Lucké, B., McCutcheon, M., and Strumia, M., *J. Exp. Med.*, 1929, 49, 779.

TABLE II. Blood Sugar and Hemolysis in Rats on a High Lard Diet Injected with Alloxan and Alloxan with Boric Acid.

	1st injection			2nd injection			3rd injection			4th injection			
Rat	Date treatment*	Hemolysis	Blood sugar 48 hr (mg %)	Date treatment	Hemolysis	Blood sugar 48 hr (mg %)	Date treatment	Hemolysis	Blood sugar 48 hr (mg %)	Date treatment	Hemolysis	Blood sugar 48 hr (mg %)	
1	1/25 A + B	—	147	2/2 A + B	+	145	2/6 A	++++	†				
2	1/25 A + B	+	213										Dead 1/28
3	1/25 A + B	+	155	2/2 A + B	±	141	2/6 A + B	—	159	2/12 A	+++	380	
4	1/26 A	+++	*										
5	1/26 A + B	+	194	2/3 A + B	±	155	2/6 A	++++	†				
6	1/27 A	+++	732										Dead 1/31
7	1/27 A + B	—	125	2/3 A + B	—	134	2/6 A + B	—	139	2/12 A + B	+	119	

* A = Alloxan, 160 mg/kg; B = Boric acid, 160 mg/kg.

† Dead within 48 hours following injection.

alone, never more than + when boric acid was given simultaneously.

Discussion. Production of diabetes is not the only known biological effect of alloxan. Before this property was known it had been studied as a capillary poison and spasmodic. Labes and Freisburger[7] made a study of the chemical properties of alloxan from this point of view. Kidney damage has been frequently observed in connection with alloxan diabetes. Houssay and Martinez[8] studied the toxicity of alloxan with reference to diet and among other findings noted that low-fat rations protected against early mortality following injection. Data in the present paper confirm this finding but indicate no difference in severity of diabetes with low or high fat in the diet (Table I). The earlier report of the authors[1] indicated another factor in alloxan effect again related to diet: marked hemolysis following alloxan injection in animals deficient in tocopherol. There was no difference in the diabetes produced in the presence or absence of tocopherol (Table I) and little difference in survival except for those tocopherol-deficient animals which died of anemia within a day or so after injection.

When boric acid was given with alloxan there was decreased effect of alloxan in the 3 factors studied: diabetes, mortality, and hemolysis. Kuhn and Quadbeck's suggestion that the increased activity of alloxan in the presence of boric acid results in its dissipation before it reaches the pancreas seems unlikely in view of the decreased effect on mortality and hemolysis. Alloxan disappears from the blood stream very rapidly[9] and the production of diabetes depends on its concentration during the first few minutes following injection. The 160 mg/kg dose of alloxan is not far above the minimum effective dose for intraperitoneal injection. Another possible mode of action of boric acid would be formation of an alloxan-boric acid complex

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[7] Labes, R., and Freisburger, H., *Arch. f. exp. Path. u. Pharmakol.*, 1930, **156**, 226.

[8] Houssay, B. A., and Martinez, C., *Science*, 1947, **105**, 548.

[9] Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, **157**, 525.

TABLE III.
Transfusion of Disintegrated White Cells.

Rabbit No.	Control WBC ×100	Max. leukopenia ×100	Time onset leukopenia (min.)	Duration leukopenia (hr)	% leukopenia	Max. leukocytosis ×100	Time onset leukocytosis (hr)	% leukocytosis
6	168	43	1	3	74	333	4	98
7	225	64	1	½	71	448	1	98
12	172	45	1	5	79	(142)*	—	—
14A	152	24	1	6	81	(176)*	—	—
14B	175	30	1	6	88	(174)*	—	—
15A	34	11	1	2	69	143	3	379
15B	183	84	1	½	97	476	1	105
16	78	16	1	5	79	(74)*	—	—
17	267	27	1	1	90	389	2	49
21	173	33	1	½	80	286	1	65
Avg	163.05	37.70	1	2.95	80.8	345.9†	2†	132†

* The figures in parentheses are those which were not considered to represent a significant leukocytosis.

† Includes only those with a significant leukocytosis.

TABLE IV.
Summary of Data.

Type of transfusion	Avg control WBC	% developing leukopenia	Avg leukopenia	Avg % drop in WBC	Avg leukocytosis	% developing leukocytosis	Avg % increase in WBC
Autotransfusion	9.175	88	2,632	69.3	18,833	56	164
Heterotransfusion	14,417	100	3,370	71 .	40,350	53	152
Disintegrated WBC's	16,305	100	3,770	80.8	34,590	60	132

the leukocyte count occurred in the other 2 animals. An initial elevation of the leukocyte count did not occur in any animal. The average time of onset of the leukopenia was 2.8 minutes, the average duration 2.2 hours and the average drop in leukocyte count 69.3% of the control count. In 9 of the 16 rabbits (56%) a subsequent leukocytosis occurred. In these rabbits the average rise in leukocytes was 164.8% of the original leukocyte count and the average time of onset was 4.3 hours after the transfusion was given.

When the cell suspension was transfused into the ear vein of another rabbit, a marked leukopenia developed rapidly in all instances (Table II). The average drop in white count was 72% of the control count, the average time of onset 2.2 minutes, and the average duration 2.2 hours. In 9 of 17 rabbits a subsequent significant rise in leukocyte count occurred. The average maximum rise in leukocyte count was 152% of the initial value and the average time of onset was 3.7 hours.

Intravenous administration of the supernatant fluid obtained by centrifugation of disintegrated leukocytes also produced a leukopenia in all instances (Table III). The average drop in white count was 80.8% of the control count, the average time of onset was 1 minute, and the average duration was 2.95 hours. A subsequent leukocytosis occurred in 6 of 10 rabbits. The average maximum rise in white count was 132% of the control count and the average time of onset of the rise in white count was 2.0 hours. The results of autotransfusion, heterotransfusion and transfusion of disintegrated white cells are summarized in Table IV.

The drop in white count was accompanied by a decrease of approximately 20% in neutrophils and a corresponding increase in the percentage of lymphocytes in the differential count. During the phase of leukocytosis there was a marked increase in the number of neutrophils (to 90% or more) with a shift to the left.

Administration of normal saline, Tyrode's solution, whole blood, heparin, killed typhoid bacilli and a solution of desoxyribosenucleic acid intravenously did not produce any sig-

TABLE I.
Autotransfusion: Transfusion of White Cells from Rabbit's Peritoneal Cavity into Ear Vein of Same Rabbit.

Rabbit No.	Control WBC ×100	Max. leukopenia ×100	Time onset leukopenia (min.)	Duration leukopenia (hr)	% leukopenia	Max leukocytosis ×100	Time onset leukocytosis (hr)	% leukocytosis
12	45	15	1	2	67	123	6	173
20	191	51	5	1	73	321	3	68
RP 1	59	12	1	4	65	(52)*	—	—
21	223	47	10	1	88	530	3	133
25	128	31	1	5	76	(100)*	—	—
26	104	44	5	5	58	(71)*	—	—
569	51	—	—	—	—	192	1	276
29	211	26	1	5	89	(82)*	—	—
31	70	18	1	½	73	148	4	116
33	134	39	1	3	70	(198)*	—	—
41	51	16	1	1	69	(50)*	—	—
43	32	7	1	1	78	81	3	149
46	30	19	1	1	38	85	6	179
48	58	32	10	1	45	(87)*	—	—
53	36	—	—	—	—	135	7	305
55	43	7	1	½	84	79	6	84
Avg	91.75	26.32	2.8	2.2	69.3	188.33†	4.3†	164.8†

* The figures in parenthesis are those which were not considered to represent a significant leukocytosis.

† Includes only those with a significant leukocytosis.

TABLE II.
Heterotransfusion: Transfusion of White Cells from Rabbit's Peritoneal Cavity into Ear Vein of Another Rabbit.

Rabbit No.	Control WBC ×100	Max. leukopenia ×100	Time onset leukopenia (min.)	Duration leukopenia (hr)	% leukopenia	Max leukocytosis ×100	Time onset leukocytosis (hr)	% leukocytosis
30	166	55	5	½	68	326	4	96
32	190	30	5	6	79	(148)*	—	—
34	67	37	5	2	43	151	3	122
40	235	54	1	2	79	748	3	218
44	268	65	1	4	75	(282)*	—	—
45	95	29	1	3	69	263	5	175
47	74	18	5	2	75	129	7	73
49	346	28	1	1	91	1262	3	235
54	158	20	1	2	87	(184)*	—	—
56	80	21	1	2	73	(88)*	—	—
57	108	28	1	4	74	(102)*	—	—
RP 9	118	51	1	2	57	194	3	64
RP 11	108	34	1	1	68	299	2	177
RP 13	119	44	1	1	62	(152)*	—	—
RP 15	81	7	1	2	79	(111)*	—	—
RP 16	93	19	1	4	74	(128)*	—	—
RP 17	103	32	5	2	69	248	3	208
Avg	144.17	33.70	2.2	2.2	72	403.50†	3.7†	152†

* The figures in parentheses are those which were not considered to represent a significant leukocytosis.

† Includes only those with a significant leukocytosis.

of the cells were mature neutrophils.

Disintegrated leukocytes were obtained by subjecting the resuspended leukocytes to supersonic vibration for 1 hour at 0°C. The material obtained was centrifuged and the supernatant fluid, free of particulate matter, was administered intravenously to rabbits.

Results. Transfusion of leukocytes into the ear vein of the same rabbit from which they had been obtained resulted in a sudden profound leukopenia in 14 of 16 rabbits (Table I). A transient unsustained drop in animals in which leukopenia has been produced by administration of leukocytes or their products, and in no animal in which leukopenia was induced with sodium citrate solution, argues against the subsequent leukocytosis being a response of the bone marrow stimulated in some manner by the leukopenia. The possibility of the existence of differences in the reaction of the recipient is indicated by the production of leukocytosis in one rabbit but not in another with the same cell suspension.

The chemical nature of the active substances present in solution of lysed leukocytes has not as yet been determined. Preliminary experiments indicate that removal of lipids, including thromboplastin, by ether extraction does not reduce its activity. Considerable loss of activity results from exposure to heating at 66°C for 1 hour.

Summary and conclusions. 1. Leukocytes were obtained in large numbers by introduction of large amounts of physiologic salt solution into the peritoneal cavity of rabbits.

2. Intravenous administration of these cells to the same or different animals was followed by very rapidly developing and severe leukopenia. A subsequent leukocytosis developed in the majority of rabbits after several hours.

3. Disintegrated leukocytes, or aqueous extract of disintegrated leukocytes produced similar results.

4. Preliminary data indicate that the leukocytes stick in the capillaries of the lung. Ether extraction of disintegrated leukocytes does not cause loss of activity but heating reduces activity.

5. These experiments indicate that unsatisfactory preservation of leukocytes in stored blood is not the reason for failure to raise leukocyte counts with transfusions, but that white blood cells contain a substance (or substances) which is capable of producing leukopenia.

We are indebted to Dr. William Holden for the assay of the thromboplastic content of leukocytes.

17057

Effect of Adrenocorticotrophic Hormone Upon Liver Fat and Urinary Phosphorus in Normal Force-Fed Rat.

Choh Hao Li, Dwight J. Ingle, Herbert M. Evans, Mildred C. Prestruda and James E. Nezamis.

From the Institute of Experimental Biology, University of California, Berkeley, and The Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

It was shown by Baker, Ingle, Li, and Evans[1] that the administration of pure adrenocorticotrophic hormone (ACTH) to force-fed male rats caused fatty infiltration of the liver as was demonstrated histologically by Sudan stains. This observation was confirmed by chemical analysis of the liver in the present study. In addition, during the administration of ACTH there was noted a significant increase in the urinary excretion of inorganic phosphorus which accompanied a rise in urinary nitrogen and that there was excretion of glucose and loss in body weight. Changes in the weights of certain organs was noted.

Methods. Male rats of the Sprague-Dawley strain were maintained on a diet of Archer Dog Pellets until they reached a weight of approximately 300 g. They were then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technic of force-feeding and the diets used were modinificant change in the leukocyte count. 10 to 15.0 cc of a 1.1% solution of sodium citrate always produced a marked leukopenia but was never followed by leukocytosis.

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[1] Baker, B. L., Ingle, D. J., Li, C. H., and Evans, H. M., *Am. J. Anat.*, 1948, **82**, 75.

Mixture of whole blood and white blood cell suspension *in vitro* resulted in the calculated rise in the total white count with no significant change in the count over a period of several hours. It was not possible to demonstrate any differences in white blood cell types by cross-matching with the sera from various recipient rabbits.

Transfusions with intact leukocytes as well as with products of leukocytic disintegration frequently resulted in sudden death, preceded by nystagmus and convulsions in all cases. Rabbits used for repeated transfusions lost weight, appeared chronically ill, and frequently died. The cellular debris from disintegrated white cells appeared to be the most toxic of the substances used. When cell suspensions contained a high percentage of cells showing toxic granulation and disintegrating cells as determined by examination of smears, toxic reactions appeared to be more common than if such were not the case. The thromboplastic activity of leukocytes disintegrated by supersonic vibration was determined. The greatest thromboplastic activity was found in the centrifuged sediment. 0.1 cc of the material was equivalent to 0.940 mg of Maltine thromboplastin; in contrast, 0.3 cc of the supernatant material from disintegrated leukocytes contained less than 0.1 mg. In spite of the high content of thromboplastin, intravenous clotting was not observed in any animal.

Because of the histamine content of leukocytes[2] the effect of intravenous histamine phosphate on the white count was investigated. 0.2 to 0.6 mg of histamine phosphate produced a mild delayed leukopenia in 3 of 10 rabbits. Neither the time of onset, the degree of leukopenia nor the duration of the leukopenia corresponded to that produced with the transfusion of intact leukocytes or the products of their disintegration. One mg of histamine intravenously resulted in sudden death with nystagmus and convulsions. The intravenous administration of an anti-histaminic (pyranisamine maleate) did not prevent the characteristic leukopenia which developed with the transfusion of a white cell suspension in 2 rabbits.

Discussion. It is evident that there is a potent substance (or substances) present in leukocytes obtained from the rabbit's peritoneal cavity which is capable of affecting the level of the leukocyte count of circulating blood. Not only do the transfused cells disappear from the circulation but white cells circulating prior to the transfusion disappear as well. This, together with the results obtained with particle-free solutions of lysed cells would indicate that the effect is more than a mere filtering of foreign bodies by the reticuloendothelial system.

The fate of the transfused cells has been studied in preliminary experiments using leukocytes labeled with radioactive phosphorus. With this method the greatest concentration of radioactivity is found in the lungs. This finding is confirmed by histologic studies of lungs removed at the time of maximum leukopenia. The lung capillaries were found to contain large numbers of leukocytes without edema or fibrin formation. The liver and spleen also contained increased numbers of leukocytes but this finding was not as marked as in the lungs.

Menkin[3,4] has described a leukopenic and leukocytic factor, as well as a toxic substance called necrosin, obtainable from inflammatory exudates. It is possible that these substances obtained from exudates are derived entirely from disintegrating leukocytes. It is noteworthy that effects obtained by administration of leukocytes produced with physiologic salt solution are similar to those obtained by exudates produced with irritants.

It cannot be stated whether leukocytosis is a response to a specific agent contained in the transfused leukocytes or their products, whether it is a response on the part of the bone marrow initiated by the leukopenia or whether it is a spontaneous variation. The fact that leukocytosis does not develop in all animals in which leukopenia has been produced by administration of leukocytes or their products, and in no animal in which leukopenia was induced with sodium citrate solution, argues against the subsequent leukocytosis being a response of the bone marrow stimulated in some manner by the leukopenia. The possibility of the existence of differences in the reaction of the recipient is indicated by the production of leukocytosis in one rabbit but not in another with the same cell suspension.

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[2] Code, C. F., *J. Physiol.*, 1937, **90**, 485.

[3] Menkin, V., *Arch. Path.*, 1946, **42**, 154.

[4] Menkin, V., *Science*, 1947, **105**, 538.

The chemical nature of the active substances present in solution of lysed leukocytes has not as yet been determined. Preliminary experiments indicate that removal of lipids. including thromboplastin, by ether extraction does not reduce its activity. Considerable loss of activity results from exposure to heating at 66°C for 1 hour.

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[1] Baker, B. L., Ingle, D. J., Li, C. H., and Evans, H. M., *Am. J. Anat.*, 1948, **82**, 75.

TABLE I.
Medium Carbohydrate Diet.

Constituent	g
Cellu flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Diet yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vit. K (2-methyl-1,4-naphthoquinone	100 mg
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc

fications of those described by Reinecke, Ball, and Samuels.[2] The diet was made according to Table I. During the period of adaptation to force-feeding the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc per day on the 5th day.

The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78°F and the humidity at 30 to 35% of saturation. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol and 1 g of citric acid per sample to insure the acidity of the urines for nitrogen analysis. The following methods of analysis were used: urinary inorganic phosphorus, Müller;[3] urinary non-protein nitrogen by the micro-Kjeldahl procedure; tissue fat and protein, Li, Simpson, and Evans.[4]

The ACTH was prepared by the method of Li, Simpson, and Evans.[5] Following control periods of 14 days, 6 experimental animals were each given 3 mg of ACTH in 7 divided injections every 2 hours during the day for a period of 10 days. One animal was killed by a feeding accident so that the final averages are based upon 5 animals. Six control animals were given injections of physiological saline. At the end of the experiment the animals were anesthetized with ether and exsanguinated. The liver was subjected to lyophilization at low temperature and was then analyzed for fat and protein. Weights were obtained on a number of organs.

Results. The data on body weight, urinary non-protein nitrogen, inorganic phosphorus and glucose are in Fig. 1, the data on organ weights in Table II, and the data on liver composition in Table III. The administration of ACTH caused a significant increase in liver fat so that all of the individual values were higher than any of the control values. The water content of the ACTH livers was correspondingly decreased. The difference between the average protein content of ACTH and control livers was too small to be considered significant.

The injections of ACTH caused a loss of weight associated with a rise in urinary nitrogen, inorganic phosphorus and glycosuria. ACTH caused an increase in the weight of the liver, hypertrophy of the adrenal glands and atrophy of the thymus. The average weights of the organs of the gastrointestinal tract and of the testes were somewhat less in the ACTH series but the kidneys and hearts were heavier. Since the numbers of animals were small the group differences cannot be

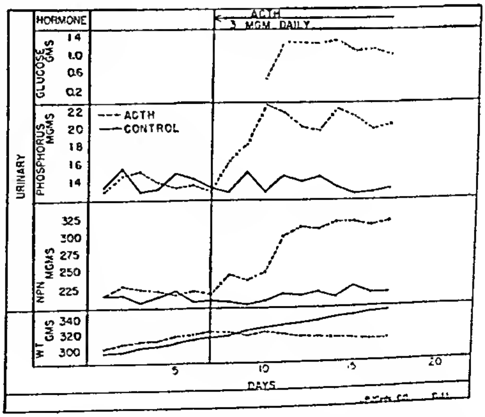

FIG. 1.

The effect of adrenocorticotrophic hormone upon body weight and some constituents of urine in the force-fed normal rat. Averages.

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[2] Reinecke, R. M., Ball, H. A., and Samuels, L. T., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 44.

[3] Müller, E., *Hoppe-Seyler's "Zeitsch. fur. Physiol. Chemie.,"* 1935, **237**, 35.

[4] Li, C. H., Simpson, M. E., and Evans, H. M.. *Growth*, 1948, **12**, 39.

[5] Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1942, **146**, 627.

TABLE II.
Organ Weight. Grams.

Organ	Adrenocorticotrophic hormone		Controls	
	Avg	Range	Avg	Range
Liver	12.17	10.11-16.21	10.27	9.50-10.80
Stomach	1.33	1.30- 1.40	1.47	1.35- 1.61
Small intestine	5.51	5.11- 5.95	6.04	5.90- 6.40
Cecum and colon	1.74	1.65- 1.80	1.94	1.70- 2.20
Kidneys	2.27	2.10- 2.49	2.01	1.86- 2.01
Testes	3.14	2.65- 3.35	3.32	3.15- 3.45
Heart	1.03	0.97- 1.05	0.94	0.93- 0.97

TABLE III.
Liver Composition of Rats Treated with Adrenocorticotrophic Hormone. Averages and standard deviations of averages.

	Body wt, g	Liver wt, g	Composition in g per 100 g wet liver		
			Water	Fat	Protein
ACTH	315 ±5.7	11.97 ±1.1	61.1 ±1.2	11.96 ±1.4	20.4 ±0.6
Control	347 ±3.1	10.27 ±0.4	67.5 ±0.4	5.83 ±0.6	21.73 ±0.6

regarded as highly reliable but there was no overlapping in individual values of the two groups in the case of the heart and kidneys and it is probable that ACTH caused these organs to gain weight.

Discussion. There are a number of experimental conditions which cause fatty livers in non-adrenalectomized animals. This response has not been described in the adrenalectomized animal,[6] although Chaikoff *et al.*[7] have reported on the production of fatty livers in hypophysectomized - thyroidectomized dogs. Hartman *et al.*[8] have prepared a fraction of adrenal cortex extracts which sustains the ability of the adrenalectomized rat to deposit fat in the liver. The relationship of adrenal cortical function to the accumulation of liver fat and to other aspects of fat metabolism is not well understood.

One of the objectives of this experiment was to study carcass fat in animals treated with ACTH without the development of glycosuria. It was not anticipated that these animals fed a medium carbohydrate diet would develop glycosuria when given ACTH. The administration of ACTH or of certain adrenal extracts and steroids either inhibits the protein anabolism or stimulates protein catabolism. If the energy represented by the missing protein is not wasted by incomplete metabolism, it must either be stored as fat (the amount of energy which can be stored as extra carbohydrate is very limited) or dissipated by a higher energy output. The effect of ACTH and of cortical hormone overdosage on energy output has not been fully studied. There are two reports[9,10] that 11-dehydrocorticosterone can cause an increase in carcass fat in the mouse. In the present study it was calculated from the extent of rise in urinary nitrogen that the administration of ACTH inhibited the accumulation of approximately 4 g of protein per rat during the 10-day period. During the same period each rat excreted an average of more than 9 g of glucose. Under these conditions an accumulation of carcass fat cannot be expected and this aspect of the problem remains for further study.

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[6] Ingle, D. J., *J. Clin. Endocrinol.*, 1943, **3**, 603.

[7] Chaikoff, I. L., Entenman, C., Rinehart, J. F., and Reichert, F. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 170.

[8] Hartman, F. A., Brownell, K. A., and Thatcher, J. S., *Endocrinology*, 1947, **40**, 450.

[9] Kendall, E. C., Josiah Macy, Jr. Foundation, Conference on Metabolic Aspects of Convalescence, Transactions of 10th Meeting, 1945, 81.

[10] Kochakian, C. D., Josiah Macy, Jr., Foundation, Conference on Metabolic Aspects of Convalescence, Transactions of 6th Meeting, 1944, 13.

The effect of ACTH in causing an increased loss of urinary phosphorus is similar to the effect of 11-dehydro-17-hydroxycorticosterone noted by Ingle and Thorn.[11]

Summary. Normal male rats were force-fed a medium carbohydrate diet. The administration of adrenocorticotrophic hormone in amounts of 3 mg per rat per day for 10 days caused an increase in liver fat, glycosuria, a rise in urinary nitrogen and inorganic phosphorus, suppression of weight gains and some changes in organ weights.

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[11] Ingle, D. J., and Thorn, G. W., *Am. J. Physiol.*, 1941, **132**, 670.

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